

Engineered Extracellular Vesicles from Antler Blastema Progenitor Cells: A Therapeutic Choice for Spinal Cord Injury

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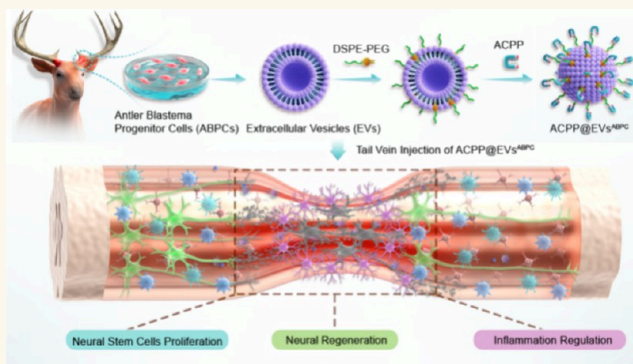
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ABSTRACT: Deer antler blastema progenitor cells (ABPCs) are promising for regenerative medicine due to their role in annual antler regeneration, the only case of complete organ regeneration in mammals. ABPC-derived signals show great potential for promoting regeneration in tissues with limited natural regenerative ability. Our findings demonstrate the capability of extracellular vesicles from ABPCs (EVs^{ABPC}) to repair spinal cord injury (SCI), a condition with low regenerative capacity. EVs^{ABPC} significantly enhanced the proliferation of neural stem cells (NSCs) and activated neuronal regenerative potential, resulting in a 5.2-fold increase in axonal length. Additionally, EVs^{ABPC} exhibited immunomodulatory effects, shifting macrophages from M1 to M2. Engineered with activated cell-penetrating peptides (ACPPs), EVs^{ABPC} significantly outperformed EVs from rat bone marrow stem cells (EVs^{BMSC}) and neural stem cells (EVs^{NSC}), promoting a 1.3-fold increase in axonal growth, a 30.6% reduction in neuronal apoptosis, and a 2.6-fold improvement in motor function recovery. These findings support ABPC-derived EVs as a promising therapeutic candidate for SCI repair.

KEYWORDS: extracellular vesicles, antler blastema progenitor cells, spinal cord injury, neural stem cells, axonal growth



Spinal cord injury (SCI) is a debilitating condition that results in irreversible harm and permanent impairment of sensory, autonomic, and motor function.^{1–3} Epidemiological data from the World Health Organization (WHO) have revealed more than 250,000 SCI patients every year, with late mortality from acute SCI ranging from 4.4% to 16.7% worldwide.^{4–6} Despite advancements in SCI management, current treatments—such as surgery, corticosteroids, and rehabilitation—offer only limited benefits. These approaches help reduce inflammation or provide supportive care but fall short of restoring lost neural function or promoting meaningful nerve regeneration.⁷ Half of the affected individuals remain paralyzed, with life expectancies of decades with permanent disabilities. Due to the limited ability of the central nervous system to repair itself following injury, re-establishment of the neural network is essential for functional recovery.⁸ Nevertheless, axonal growth after SCI is limited by the poor intrinsic regenerative capacity of neurons and the hostile extrinsic injury environment that compromises inflammation, myelin-associated inhibitors, and glial scars.^{9,10}

Therefore, it is crucial to target the diminished intrinsic regenerative ability of neurons and relieve extrinsic inhibitory cues within the environment.^{10,11} Hence, developing a strategy that tackles both challenges may enhance the current state of SCI treatment.

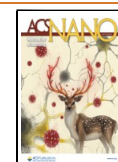
Studies on SCI treatment have shown promising results with stem cell-based therapies.¹² Mesenchymal stem cells (MSCs) are particularly noteworthy among existing stem cell options for SCI treatment because they are typically obtained from postnatal tissues and have fewer safety and ethical concerns.¹³ To date, MSCs sourced from diverse origins, such as adipose tissue, bone marrow, and umbilical cord, have been shown to

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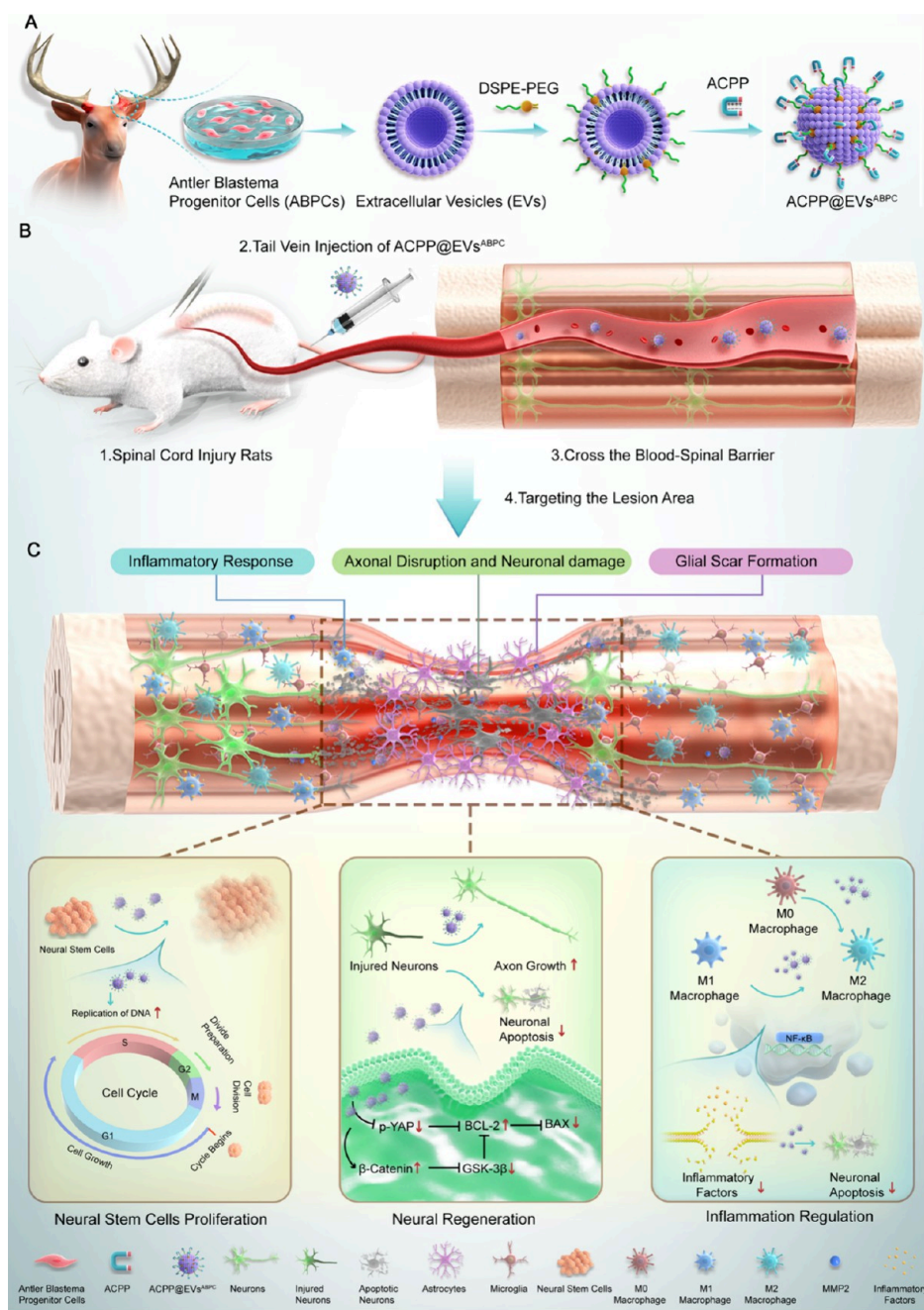
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Scheme 1. Schematic Illustration of Antler Blastema Progenitor Cell-Derived Extracellular Vesicles and Their Therapeutic Role in Spinal Cord Injury (SCI)^a



^a(A) Isolation and modification of EVs^{ABPC}, derived from antler blastema progenitor cells (ABPCs) isolated from deer antler, were modified with DSPE-PEG and activatable cell-penetrating peptides (ACPP) to enhance their targeting ability at the SCI lesion site. (B) Intravenous injection of ACPP@EVs^{ABPC} in spinal cord injury (SCI) rats enables them to cross the blood-spinal cord barrier (BSCB) and selectively accumulate at the injury site. (C) SCI is characterized by an acute inflammatory response, axonal disruption and neuronal damage, and glial scar formation, which collectively inhibit neural repair and functional recovery. At the lesion site, ACPP@EVs^{ABPC} promote neural stem cell proliferation by enhancing cell cycle progression through the S phase, facilitate axonal growth, and reduce neuronal apoptosis via modulation of the Wnt and Hippo signaling pathways. Additionally, EVs^{ABPC} regulate inflammation by promoting macrophage polarization from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype through the NF- κ B signaling pathway, thereby supporting tissue repair and functional recovery.

improve the outcome of SCI.^{14,15} Pro-regenerative factors from MSCs can enhance the intrinsic neuronal regenerative effects of insulin-like growth factor-1 (IGF-1) and nerve growth factor (NGF).^{16,17} In addition, MSCs help establish a beneficial microenvironment that facilitates axonal regrowth by ameliorating neuroinflammation and inhibiting neuron apoptosis.¹⁸

Nevertheless, MSCs typically undergo dynamic cellular senescence during multipassage cultivation *in vitro*.^{19,20} Additionally, MSCs are generally obtained from mammalian postnatal tissues, which show relatively constrained regenerative ability and typically contribute to partial tissue regeneration in mammals, resulting in a ceiling effect in the

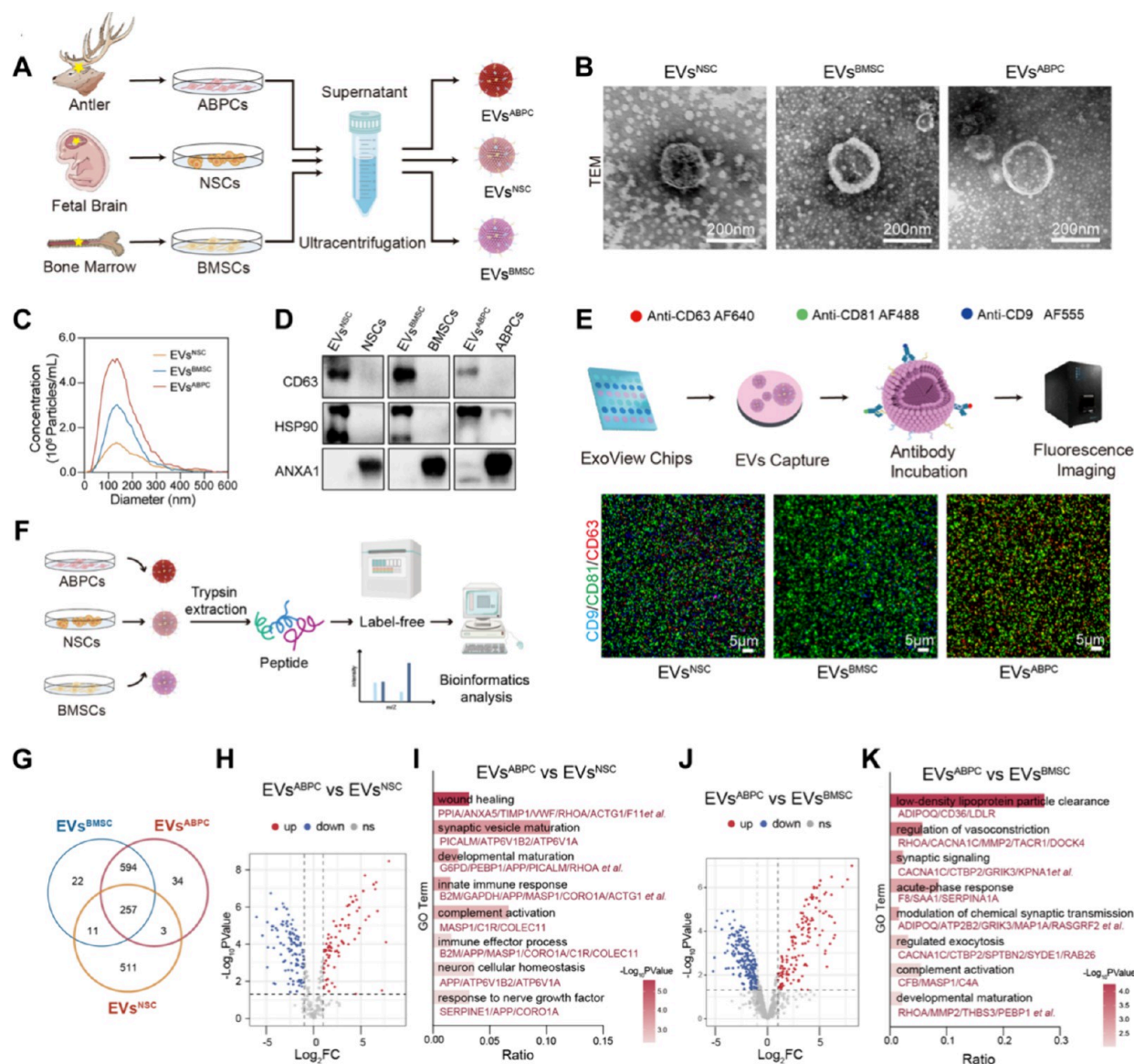


Figure 1. Characterization and proteomic analysis of EVs^{ABPC}, EVs^{NSC}, and EVs^{BMSC}. (A) Schematic depicting the extraction process of EVs from ABPCs, NSCs, and BMSCs. The supernatants from the cultured cells were collected and subjected to ultracentrifugation to isolate the EVs. (B) TEM images validated the morphology of EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC}. Scale bar, 200 nm. (C) Quantification and size analyses of EVs^{ABPC}, EVs^{NSC}, and EVs^{BMSC} using NTA. (D) Western blot analysis of EVs marker proteins (CD63, HSP90, and ANXA1). An equal amount (2 μ g) of protein in EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC} was loaded in each lane, using NSC, BMSC, and ABPC lysates as controls for surface markers. (E) ExoView analysis of EVs^{ABPC}, EVs^{NSC}, and EVs^{BMSC}. Representative composite images show EV markers CD9 (blue), CD81 (green), and CD63 (red). Scale bar, 5 μ m. (F) Schematic illustration of the proteomic analysis workflow. EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC} were subjected to trypsin digestion and analyzed using a label-free peptide quantification method, followed by bioinformatic analysis. (G) Venn diagram showing the overlap of identified proteins among EVs^{ABPC}, EVs^{NSC}, and EVs^{BMSC}. (H) Volcano map indicating DEPs between EVs^{ABPC} and EVs^{NSC}. Upregulated proteins are denoted using red dots, while downregulated proteins are indicated using blue dots; nonsignificant proteins are represented using gray dots. (I) GO term enrichment analyses depicting enriched biological processes upregulated between EVs^{ABPC} and EVs^{NSC}. (J) Volcano plot showing DEPs between EVs^{ABPC} and EVs^{BMSC}. (K) GO term enrichment analyses depicting enriched biological processes upregulated between EVs^{ABPC} and EVs^{BMSC}.

promotion of tissue regeneration.²¹ Therefore, the discovery of novel MSCs with robust regenerative potential in mammals may revolutionize stem cell studies within the domain of regenerative medicine.

In a recent study, we discovered a new type of MSCs in the growing deer antler, denoted antler blastema progenitor cells (ABPCs).²² ABPCs and their progeny drive bony antler growth at an astonishing rate. The growth rate of antlers is up to 2.75 cm per day, allowing them to achieve a mass of up to 15 kg and a length of 120 cm within three months, which is the

fastest organ growth recorded in mammals.²³ Intriguingly, ABPCs exhibit negligible signs of senescence and retain robust proliferative and regenerative capacities after 50 passages *in vitro*.²² This is in contrast to commonly used MSCs, such as those from the umbilical cord, adipose tissue, and bone marrow, which generally undergo obvious cellular senescence after 10–15 passages of *in vitro* culture.²⁴ To our knowledge, ABPCs are the only MSCs from postnatal mammalian tissue that support complete organ regeneration in mammals, highlighting their robust pro-regenerative ability in promoting

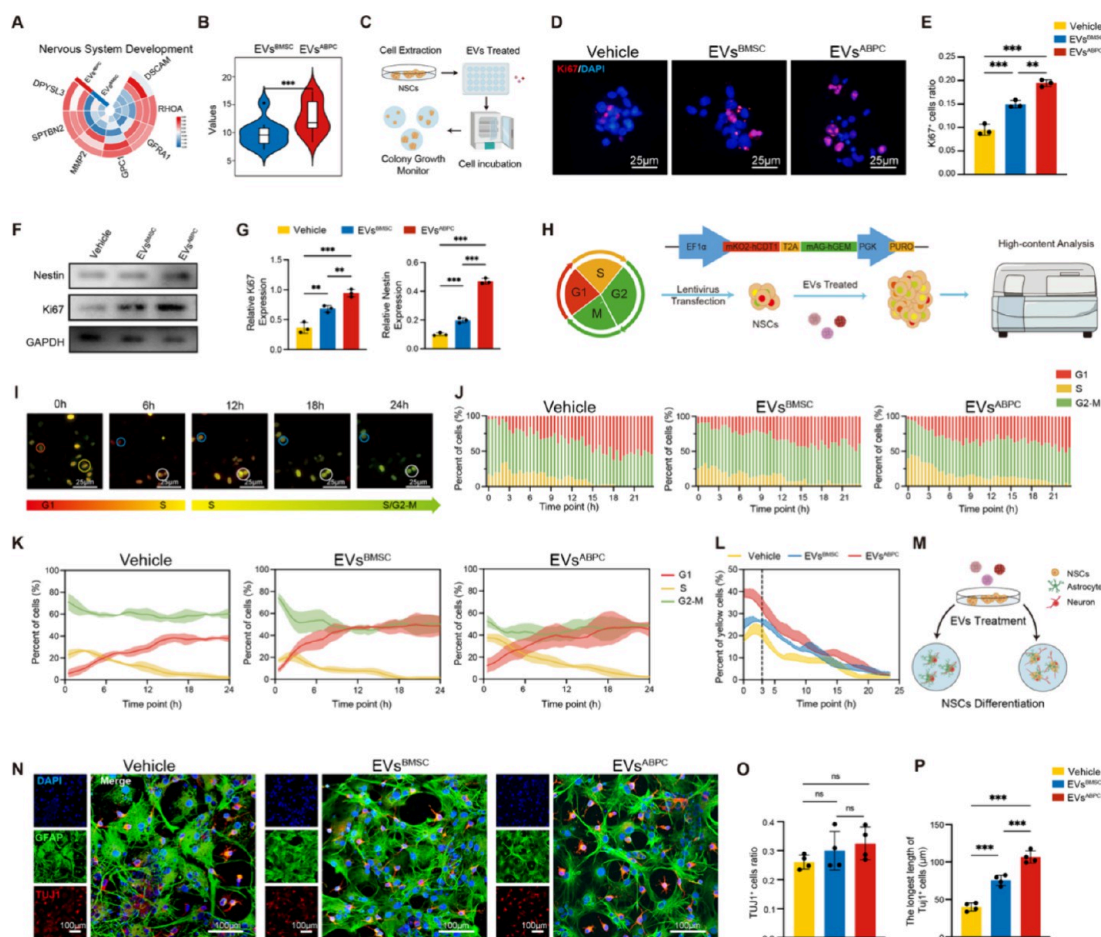


Figure 2. EVs^{ABPC} enhanced NSC proliferation *in vitro*. (A) Circular heatmap showing DEPs related to nervous system development between EVs^{ABPC} and EVs^{BMSC}. (B) Violin plot depicting proteins related to nervous system development regulation in EVs^{ABPC} and EVs^{BMSC}. Central box in each violin represents the interquartile range (IQR). Statistical significance between groups was determined using an unpaired Student's *t*-test. ****P* < 0.001. (C) Schematic of EVs^{ABPC} and EVs^{BMSC} effects on NSCs proliferation. (D–E) Ki67 immunofluorescence staining (D) and quantification (E) in primary NSCs treated with vehicle, EVs^{BMSC}, or EVs^{ABPC} (*n* = 3). Scale bar, 25 μ m. (F) Western blot detection of Nestin, Ki67, and GAPDH protein levels in the vehicle, EVs^{BMSC}, and EVs^{ABPC} groups. (G) Quantification of Nestin and Ki67 protein levels after different treatments (*n* = 3). (H) Schematic representation of FUCCI-based cell cycle evaluation. (I) Representative cell cycle images. Circle indicates tracked cell transitioning from G1 to G2/M within 24 h. Scale bar, 25 μ m. (J) Bar graphs depicting the proportion of cells in different cell cycle phases over time under different treatments. (K) Line graphs showing dynamic variations in the proportion of cells in G1, S, and G2-M phases over time under different treatments (*n* = 3). Solid lines represent mean values for each phase, while shaded areas indicate standard deviation (SD). (L) Comparison of dynamic changes in the proportion of NSCs in S phase at different time points over 24 h under different treatments (*n* = 3). (M) Schematic illustration of evaluating effects of EVs^{ABPC} on NSC differentiation. (N) Representative immunofluorescence images showing expression of GFAP and TUJ1 in NSCs treated with vehicle, EVs^{BMSC}, and EVs^{ABPC} for 2 d. Scale bar, 100 μ m. (O) Measurement of proportion of TUJ1⁺ cells in each treatment group (*n* = 4). (P) Measurement of longest neurite length in TUJ1⁺ cells from each treatment group (*n* = 4). (E, G, O, and P) Statistical data are presented as mean $\pm$ SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni's posthoc test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. ns: not significant.

regeneration.^{25,26} In addition, ABPCs have immunomodulatory, antiapoptotic, and anti-inflammatory properties consistent with the inherent nature of MSCs.²⁷

These unique characteristics make ABPCs appealing for promoting tissue regeneration, particularly for poorly regenerated tissues such as the spinal cord. However, direct injection of ABPCs into the human body is not clinically acceptable because of ethical and safety concerns.²⁷ Extracellular vesicles (EVs) are nanosized membranous vesicles that transport different informative cargoes (including lipids, proteins, metabolites, and nucleic acids) from their parent cells.²⁸ Preclinical and clinical research has suggested that stem cell-derived EVs provide clinical benefits similar to those of their donor cells without the biosafety concerns inherent to stem

cell therapy.²⁹ Therefore, EV-based therapy is an ideal alternative to stem cell therapy in medical applications.^{30,31} In this study, we addressed the gap in knowledge regarding the role of EVs derived from ABPCs (EVs^{ABPC}) in SCI repair. EVs^{ABPC} are uniquely advantageous as they retain potent regenerative ABPC signals, which are not present in EVs from more commonly used stem cells like bone marrow stem cells (BMSCs) and neural stem cells (NSCs). Additionally, to overcome the challenges of targeted delivery in SCI, we engineered EVs^{ABPC} with activated cell-penetrating peptides (ACPP), enhancing their localization to the injury site. By comparing EVs^{ABPC} to BMSC- and NSC-derived EVs (EVs^{BMSC}; EVs^{NSC}), our study demonstrates how this innovative approach can more effectively promote neural

regeneration, modulate the immune response, and improve functional recovery in SCI patients. The workflow of this study is illustrated in Scheme 1.

RESULTS

Isolation and Characterization of EVs^{ABPC}. The periodic rapid growth of antlers is a meticulous process involving osteogenesis, neurogenesis, and angiogenesis, partly attributable to bioactive cues from ABPCs.^{22,32} To avoid ethical and safety concerns of xenogeneic ABPCs, EVs were purified from ABPCs (EVs^{ABPC}), which inherit a multitude of biological signals from their originating cells.²⁷ Their features were characterized and compared with those of EVs from the most widely used NSCs (EVs^{NSC}) and BMSCs (EVs^{BMSC}) extracted from Sprague–Dawley (SD) rats (Figure 1A). EVs^{ABPC} exhibited analogous morphology, surface markers, and size distribution to EVs^{NSC} and EVs^{BMSC} (Figure 1B–D). Transmission electron microscopy (TEM) revealed the characteristic cup-shaped or spheroidal morphology of all EVs (Figure 1B). Nanoparticle tracking analysis (NTA) revealed that the overall particle size distribution profiles of EVs from the three different sources were in a similar range, with a primary peak at an approximate diameter of 154.4 nm (Figure 1C). Protein content-based characterization of the three types of EVs using Western blotting validated the elevated expression of EVs marker proteins CD63 and HSP90 while showing negative expression of the microvesicle-specific marker ANNEXIN A1 (ANXA1; Figure 1D). To ensure precise measurements and mitigate non-EV contaminants, we used the ExoView platform to characterize single EVs in a multiplex manner.³³ The captured EVs were labeled with fluorescent antibodies targeting CD9, CD81, and CD63. All EVs expressed the tetraspanin markers CD81, CD9, and CD63 (Figure 1E), confirming the uniformity of surface markers across the EVs.

Further proteomic analysis was performed to characterize the composition of the different EVs using liquid chromatography–tandem mass spectrometry (LC–MS/MS; Figure 1F). Principal component analysis (PCA) indicated that the three biological replicates of each EV were homogeneous, confirming the repeatability and reliability of the data (Figure S1A). Venn diagrams demonstrated that among the 1432 proteins identified, 257 overlapped with all EV types (Figure 1G). A unique proteomic profile was identified in EVs^{ABPC} via Gene Ontology (GO) analysis compared to EVs^{NSC}, in which 79 upregulated proteins were involved in wound healing (including PPIA, ANXA5, and TIMP1), synaptic vesicle maturation (including PICALM, ATP6 V1B2, and ATP6 V1A), immune response (including B2M, MASP1, and CORO1A), neuronal cellular homeostasis (including APP, ATP6 V1B2, and ATP6 V1A), and response to growth factors (including SERPINE1, APP, and CORO1A; Figure 1H–I; $|\log_2(\text{FC})| > 1$ and $P < 0.05$). Furthermore, compared to EVs^{BMSC}, 139 upregulated proteins were identified in EVs^{ABPC}, which were related to synaptic signaling (including CACNA1C, CTBP2, and GRIK3), acute-phase response (including F8, SAA1, and SERPINA1A), and developmental maturation (including RHOA, THBS3, and PEBP1; Figure 1J–K, $|\log_2(\text{FC})| > 1$, and $P < 0.05$). Selected proteins, including RHOA, PICALM, and B2M, identified as upregulated and associated with wound healing pathway proteins were further validated using Western blot to strengthen the credibility of the proteomic findings (Figure S1B,C). Subsequent Rectome pathway knowledgebase enrichment analysis indicated that

upregulated differentially expressed proteins (DEPs) participated in axon guidance and nervous system development compared to EVs^{NSC} and EVs^{BMSC} (Figure S1D,E). Collectively, EVs^{ABPC} contained a variety of unique biological factors that favor neurogenesis, highlighting their therapeutic potential in addressing neurological injuries and disorders.

EVs^{ABPC} Enhanced NSC Proliferation In Vitro. Proteomic analysis revealed marked enrichment of proteins associated with nervous system development (including RHOA, DSCAM, and GFRA1) within EVs^{ABPC} compared to that within EVs^{BMSC} (Figure 2A–B). Given the crucial role of NSCs in nervous system development and repair,³⁴ we investigated the impact of EVs^{ABPC} on NSCs, with a particular focus on NSC proliferation and differentiation. We first extracted NSCs from the cerebral cortex of E14 SD rats and characterized them using the specific markers Nestin and SOX2 (Figure S2A). EVs labeled with PKH26 were observed within the soma and processes of NSCs, as shown by the colocalization of PKH26 and Nestin staining in Figure S2B. This indicates successful internalization and distribution of EVs within the NSCs. Subsequently, NSCs were treated with either EVs^{ABPC} or EVs^{BMSC}, using basal culture medium as vehicle control (Figure 2C). Upon treatment with EVs^{ABPC}, the proportion of Ki67-positive cells significantly increased ($19.5 \pm 0.7\%$). Compared to the EVs^{BMSC} and vehicle groups, 1.3-fold and 2.1-fold increases were observed, respectively, suggesting a stronger ability of EVs^{ABPC} to promote the proliferation of NSCs (Figure 2D–E). Western blot analysis confirmed these results, demonstrating markedly elevated levels of Ki67 and Nestin in the EVs^{ABPC} group compared to those in the other groups (Figure 2F–G).

We used a fluorescent ubiquitination-based cell cycle indicator (FUCCI) system to observe single-cell behaviors with precise spatiotemporal resolution. This system employs genes linked to fluorescent reporters that emit different colors specific to each cell cycle phase, allowing real-time imaging of cell cycle phases (Figure 2H).³⁵ We induced the expression of FUCCI reporters in NSCs via lentiviral transduction. Cell populations with red, yellow, or green light correspond to cells in the quiescent G1 phase, early S-phase, or proliferating late-S/G2-M phases, respectively (Figure 2I). A representative example of a cell transitioning from the G1 (red) to the G2-M phase (green) is shown in Figure 2I. In each group, we observed synchronized oscillations in the proportions of cells in the G1 (red), S (yellow), and G2-M (green) phases as the overall cell population cycled over time, reflecting typical cell cycle dynamics (Figure 2J–K).³⁶ The proportion of cells in the S phase (yellow) accumulated over 24 h in the EVs^{ABPC} group, demonstrating a marked increase compared to the other groups (Figure 2L).

Next, we explored the effects of EVs^{ABPC} on NSC differentiation (Figure 2M). Immunostaining was performed for specific markers of neurons and astrocytes using β III-tubulin (TUJ1) and glial fibrillary acidic protein (GFAP), respectively, to examine the differentiation ratio of NSCs into neurons and astrocytes (Figure 2N). No significant differences were observed in the proportions of TUJ1⁺ and GFAP⁺ cells between the groups (Figure 2N–O and S2C; $P > 0.05$), indicating a negligible effect of EVs^{ABPC} on the differentiation of NSCs. Nevertheless, we observed a marked rise in the length of TUJ1⁺ cells in the EVs^{ABPC} group ($106.5 \pm 8.13 \mu\text{m}$), which was 2.6-fold greater than that in the vehicle group and 1.4-fold that in the EVs^{BMSC} group (Figure 2P, S2D). This finding

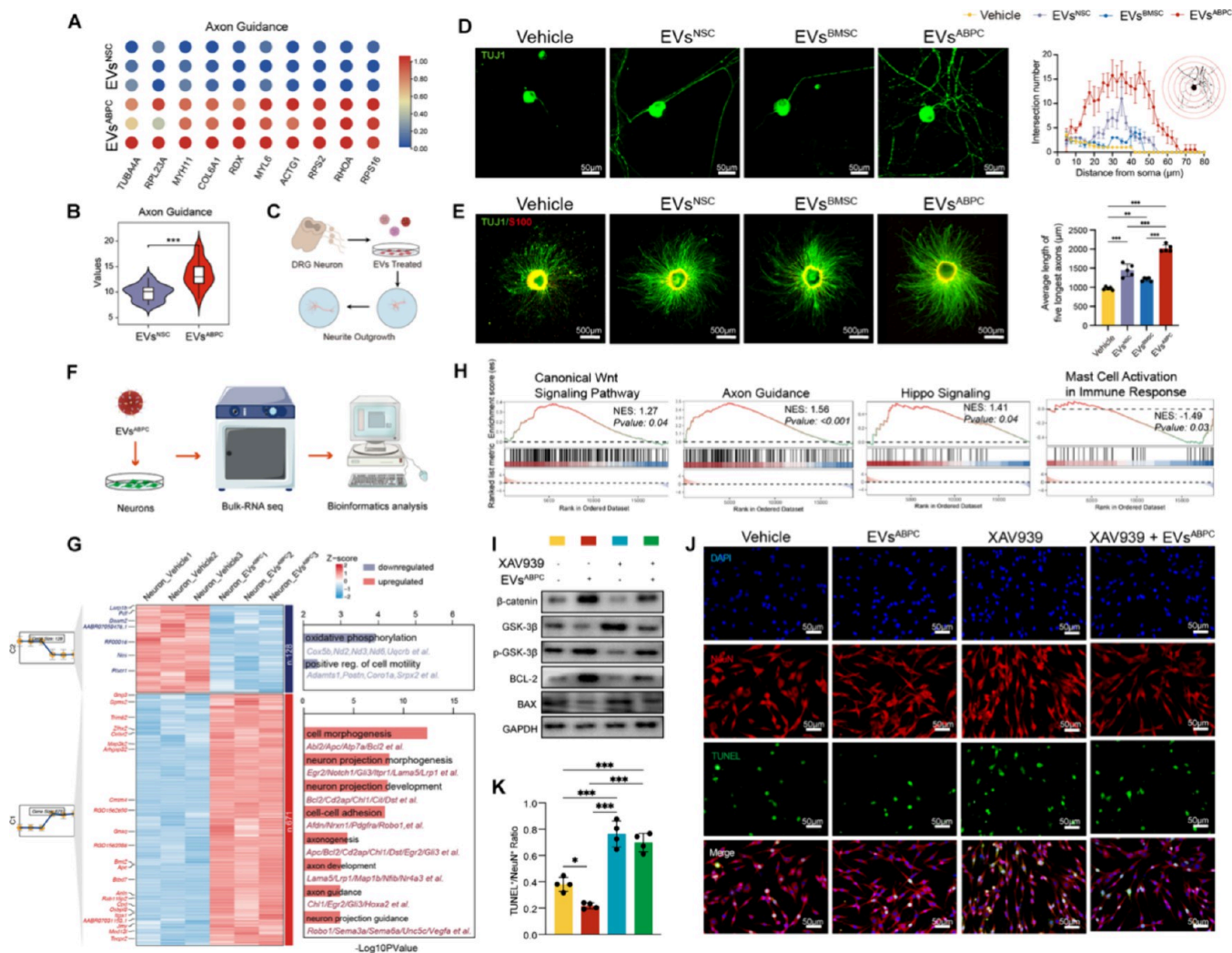


Figure 3. EVs^{ABPC} enhanced neurite outgrowth and reduce neuronal apoptosis. (A) Heatmap of DEPs related to axon guidance in EVs^{ABPC} and EVs^{NSC}. (B) Violin plot of axon guidance proteins in EVs^{NSC} and EVs^{ABPC}. Central box in each violin represents IQR. Statistical significance between groups was determined using an unpaired Student's *t*-test. ****P* < 0.001. (C) Schematic of EVs^{NSC}, EVs^{BMSC}, or EVs^{ABPC} effects on DRG neurite outgrowth. (D) Representative images (left) showing TUJ1-stained DRG neurons treated with vehicle, EVs^{NSC}, EVs^{BMSC}, or EVs^{ABPC} for 48 h. Scale bar, 50 μ m. Sholl analysis (right) demonstrates the effects of different treatments on neurite branching complexity in DRG neurons. Representative Sholl plots were shown as insets, which were normalized against the vehicle group (*n* = 3). (E) Images depicting TUJ1 (green) /S100 (red) staining (left) in DRG explants treated with vehicle, EVs^{NSC}, EVs^{BMSC}, or EVs^{ABPC} for 48 h. Scale bar, 500 μ m. Quantification of the mean length of the five longest axons in DRG explants (right) under different treatments (*n* = 5). (F) Workflow of RNA sequencing (RNA-seq) analysis. (G) RNA-seq analyses of regulated pathways in Neurons_Vehicle vs Neurons_EVs^{ABPC}. Left: Up- and downregulated genes. Middle: DEG expression heatmap. Right: Functional enrichment. (H) GSEA of biological pathways including Wnt signaling, axon guidance, Hippo signaling, and mast cell activation. (I) Western blot of key proteins in Wnt pathways in neurons treated with EVs^{ABPC} and the Wnt inhibitor XAV939. (J) Representative images of TUNEL staining in neuron cultures. Red indicates TUNEL-positive apoptotic cells, and green indicates NeuN-positive neurons. Scale bar, 50 μ m. (K) Quantification of apoptosis in neuron culture. All statistical data are represented as mean $\pm$ SD. (E and K) Statistical analyses were performed using one-way ANOVA with Bonferroni's posthoc test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. ns: not significant.

suggested that EVs^{ABPC} could promote the outgrowth of neurite neurons upon their differentiation from NSCs rather than affecting the lineage specification of NSCs.

EVs^{ABPC} Promoted Neurite Outgrowth and Branching. EVs^{ABPC} contained abundant proteins associated with axon guidance (including RPS2, ACTG1, and RHOA) in contrast to EVs^{NSC}, highlighting their potential to drive axon growth (Figure 3A–B). Therefore, dorsal root ganglion (DRG) neurons were utilized to evaluate the potential impacts of EVs^{ABPC} on axonal outgrowth (Figure 3C). Intriguingly, different EVs increased the complexity of neurite branching, which was associated with neural circuit development,

including the establishment of synapses and guidance of axons.³⁷ Sholl analysis demonstrated that in DRG neurons, the branching complexity in the EVs^{ABPC} group was approximately 1.5-fold, 2.7-fold, and 4.1-fold higher than that in the EVs^{NSC}, EVs^{BMSC}, and vehicle groups, respectively (Figure 3D). Upon treatment with EVs^{ABPC} for 48 h, the axon length of neurons significantly increased, reaching a maximum of 5.2-fold that of the vehicle, 1.3-fold that of EVs^{NSC}, and 2.2-fold that of the EVs^{BMSC} (Figure S3A) groups. In DRG explants, EVs from different sources showed a notable elevation in the mean length of the five longest axons in comparison to the vehicle group. The average length of DRG explants was the highest in

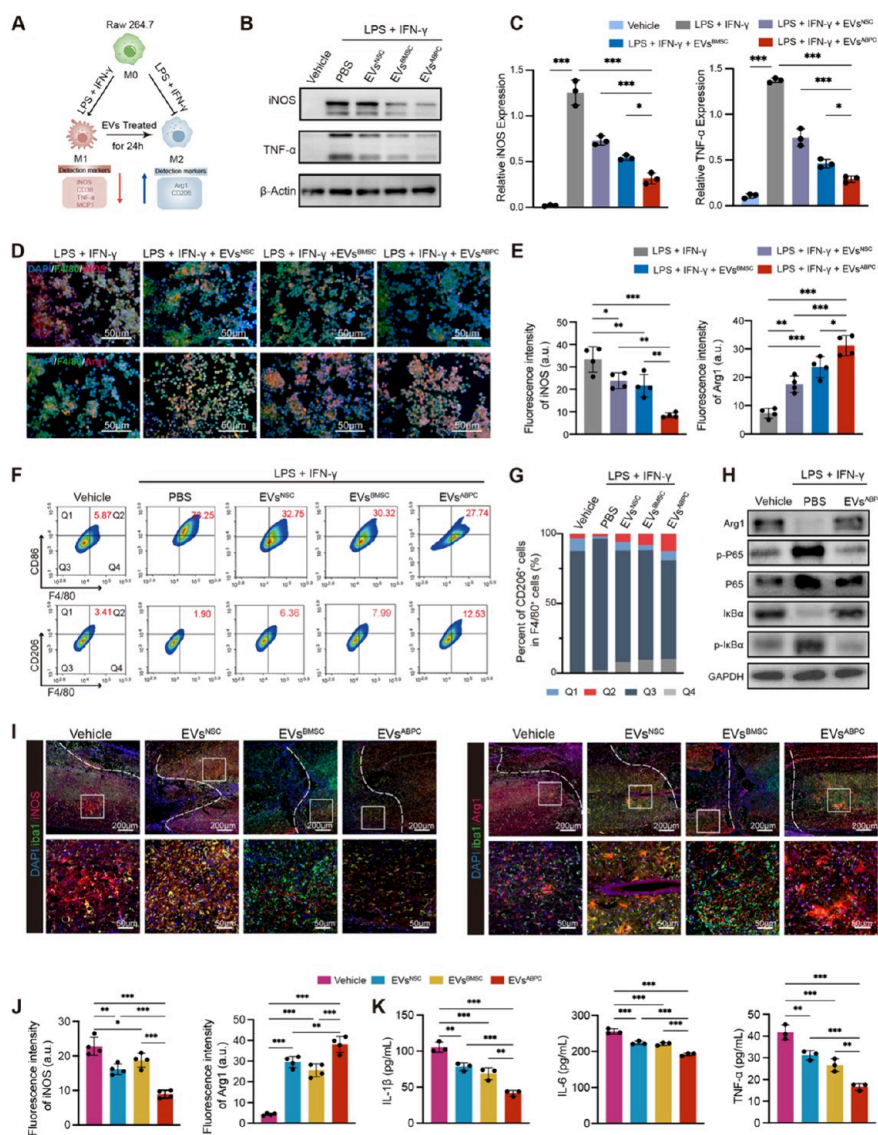


Figure 4. EVs^{ABPC} exhibited robust immunomodulatory ability *in vitro* and *in vivo*. (A) Schematic of evaluating the immunomodulatory effect of EVs on macrophage polarization in RAW264.7 cells. (B) Western blot showing proinflammatory factors iNOS and TNF- α in RAW 264.7 cells treated with LPS + IFN- γ , with or without different EVs. (C) Quantification of the relative iNOS and TNF- α expression levels in macrophages ($n = 3$). (D) Immunofluorescence images showing effects of EVs on macrophage polarization. Top: iNOS (red), F4/80 (green), DAPI (blue). Bottom: Arg1 (red), F4/80 (green), DAPI (blue). Scale bar, 50 μ m. (E) Quantification of the fluorescence intensity of iNOS and Arg1. (F) Flow cytometric analysis showing the percentage of CD86⁺ and CD206⁺ cells in F4/80⁺ gated macrophages after different treatments. (G) Quantification of the percentage of CD206⁺ cells (M2) in F4/80⁺ macrophages (primarily in Q2). (H) Western blot showing Arg1, I κ B α , p-I κ B α , p-P65, and P65 in macrophages treated with LPS + IFN- γ , with or without EVs^{ABPC}. (I) Immunofluorescence images showing effects of EVs on microglial polarization in the spinal cord at 14 dpi. Left: iNOS (red), Iba1 (green), DAPI (blue). Right: Arg1 (red), Iba1 (green), DAPI (blue). Scale bar, 200 μ m. Insets show higher magnification of boxed areas. Scale bar, 50 μ m. (J) Quantification of the fluorescence intensity of iNOS⁺ and Arg1⁺ microglia in the spinal cord ($n = 4$). (K) ELISA assay assessing spinal proinflammatory cytokine IL-1 β , IL-6, and TNF- α levels following different treatments. ($n = 3$). (C, E, J, and K) All statistical data are represented as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA with Bonferroni's posthoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns: not significant. a.u.: arbitrary unit.

the EVs^{ABPC} group ($2,017.5 \pm 95.6 \mu$ m), followed by EVs^{NSC} ($1,445.8 \pm 164.9 \mu$ m), EVs^{BMSC} ($1,205.6 \pm 46.9 \mu$ m) groups, and the lowest in the vehicle group ($969.3 \pm 40.0 \mu$ m; Figure 3E). Furthermore, in DRG explants, EVs^{ABPC} significantly increased branching complexity, which was 2.5-fold, 2.3-fold, and over 3-fold higher than that in the EVs^{NSC}, EVs^{BMSC}, and vehicle groups, respectively (Figure S3B). Collectively, the vehicle group had the shortest neurite outgrowth and the lowest number of branches in both the DRG neurons and explants. The EVs^{NSC} and EVs^{BMSC} groups exhibited moderate

increases in neurite outgrowth and branching. Notably, the EVs^{ABPC} group exhibited the strongest effects, with the most extended neurites and branches. These results demonstrated that EVs^{ABPC} outperformed the other EVs in establishing neuronal branching complexity, highlighting their potential for establishing synaptic connections.

Functional deficits following SCI primarily stem from the depletion of mature neurons, a consequence directly linked to apoptotic processes in neural cells.³⁸ We examined the effects of different EVs on Ventral spinal cord (VSC4.1) neurons.

Neuronal apoptosis was induced using lipopolysaccharide (LPS), a well-established cell model of neuronal apoptosis in SCI.³⁹ We found that EVs from different stem cells rescued neuronal apoptosis induced by LPS ($67.34 \pm 9.8\%$), with the strongest ability observed in EVs^{ABPC}, which decreased apoptosis to $15.33 \pm 2.9\%$. EVs^{NSC} and EVs^{BMSC} also demonstrated significant effects, although to a lesser extent, reducing apoptosis to approximately $35.12 \pm 3.3\%$ and $29.14 \pm 8.8\%$, respectively (Figure S3C–D). This aligns with previous findings that stem cell-derived EVs can suppress neuronal apoptosis following SCI.⁴⁰

We further examined the underlying mechanisms involved in the positive effects of EVs^{ABPC} on neurite outgrowth and branching using RNA sequencing (RNA-Seq) (Figure 3F). The PCA plot showed the clustering of neurons treated with EVs^{ABPC} compared to the vehicle group, confirming the repeatability and reliability of the data (Figure S4A). The heatmap and volcano plot (Figure 3G and S4B) revealed that 799 genes exhibited significant differential expression following EVs^{ABPC} treatment. Among these, 671 genes showed increased expression, whereas 128 genes exhibited decreased expression ($|\log_2(\text{FC})| > 0.58$ and $P < 0.05$). GO enrichment analysis revealed that the upregulated differentially expressed genes (DEGs) were associated with neuron projection development (including Bcl2, Cd2ap, and Dst), axonogenesis (including Apc, Bcl2, and Cd2ap), and axon guidance (including Egr2, Gli3, and Hoxa2), which are pivotal for nerve regeneration (Figure 3G).

Gene set enrichment analysis (GSEA) in EVs^{ABPC}-treated neurons revealed significant enrichment in multiple biological pathways, including the Hippo signaling pathway, canonical Wnt signaling pathway, axon guidance pathway, and mast cell activation pathway related to immune response (Figure 3H). Among the enriched pathways identified, the Hippo and Wnt signaling pathways were of particular interest due to their established roles in promoting cell survival and regeneration.^{41,42} Since treatment with EVs^{ABPC} alone significantly reduced neuronal apoptosis, to further investigate this hypothesis, neurons were pretreated with the Wnt pathway inhibitor XAV939 to suppress Wnt signaling activity (Figure 3I).⁴³ XAV939 treatment significantly suppressed the expression of beta-catenin (β -catenin) and increased the levels of GSK-3 β , indicating inhibited Wnt signaling activity (Figure S4C–E). Further analysis showed that, compared to the EVs^{ABPC} group, the levels of the antiapoptotic protein BCL-2 were significantly decreased, while the levels of the proapoptotic protein BAX were markedly increased in the XAV939 group. However, subsequent treatment with EVs^{ABPC} in XAV939-pretreated neurons (XAV939 + EVs^{ABPC} group) partially restored the expression of BCL-2 and significantly reduced the levels of BAX compared to the XAV939 group alone (Figure S4F–G). Additionally, TUNEL and NeuN immunofluorescence staining showed that the TUNEL/NeuN double-positive cell ratio was significantly elevated in the XAV939-pretreated group. Although this ratio decreased following subsequent EVs^{ABPC} treatment, it remained higher than that in the group treated with EVs^{ABPC} alone, further supporting the involvement of the Wnt pathway in the antiapoptotic effects of EVs^{ABPC} (Figure 3J–K).

To explore the role of the Hippo signaling pathway in the effect of EVs on neural apoptosis, we used the Hippo pathway activator Verteporfin (VTP) to enhance the activity of the Hippo pathway.⁴⁴ VTP treatment alone resulted in a marked

increase in phosphorylated YAP (p-YAP), accompanied by a decrease in BCL-2 levels and an increase in BAX levels (Figure S4H), indicating enhanced neuronal apoptosis due to Hippo pathway activation. Subsequent treatment with EVs^{ABPC} in VTP-treated neurons led to a reduction in p-YAP levels, an increase in BCL-2 levels, and a decrease in BAX levels (Figure S4H–K), suggesting that EVs^{ABPC} counteracted the proapoptotic effects induced by Hippo pathway activation. Collectively, these findings indicated that EVs^{ABPC} supported neuronal survival by regulating both the Wnt and Hippo signaling pathways.

EVs^{ABPC} Exhibited Robust Immunomodulatory Properties. EVs derived from MSCs exhibit immunomodulatory properties.⁴⁵ Given that ABPCs are a unique type of MSCs, a variety of molecules related to the modulation of inflammatory responses, such as VWF, TIMP, and COL6A1, were identified in EVs^{ABPC} (Figure S5A). In addition, the score for the gene set of the innate immune system was significantly higher in EVs^{ABPC} than in EVs^{BMSC} and EVs^{NSC} (Figure S5B), suggesting a potentially stronger immunomodulatory ability of EVs^{ABPC}.

Next, we investigated the immunomodulatory properties of EVs^{ABPC}, focusing on the shift of macrophages from a proinflammatory (M1) to a tissue-repairing (M2) phenotype, given their pivotal role in immunoregulation and nerve regeneration (Figure 4A). Upon treatment with lipopolysaccharide (LPS) and Interferon-gamma (IFN- γ), we observed a marked elevation in proinflammatory factors in RAW264.7 macrophages, including inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF- α). This finding suggested that a combination of LPS and IFN- γ was sufficient to switch M0 macrophages into the proinflammatory M1 phenotype (Figure 4B). Notably, the expression of these proinflammatory factors was significantly reduced following EV application, with EVs^{ABPC} showing the most pronounced effect (Figure 4C). Immunofluorescence staining showed that PKH26-labeled EVs^{ABPC} were internalized by macrophages and distributed within the soma of M1 macrophages (Figure S5C). In addition, the fluorescence intensity of iNOS⁺ cells (M1) significantly decreased after EVs^{ABPC} treatment, showing a reduction to approximately 14.1% of the level observed in the LPS + IFN- γ group, 35.4% of the level observed in the EVs^{NSC} group, and 39.1% of the level observed in the EVs^{BMSC} group. Conversely, the EVs^{ABPC}-treated group exhibited a remarkable increase in the fluorescence intensity of Arg1⁺ cells (M2), which was approximately 2.7-fold greater than that in the LPS + IFN- γ group (Figure 4D–E). An identical trend was observed using fluorescence-activated cell sorting (FACS). Upon treatment with LPS + IFN- γ , the CD206⁺/F4/80⁺ polarization ratio (M2/M0) increased from 1.90% (vehicle) to 5.87% (EVs^{NSC}), 7.99% (EVs^{BMSC}), and further to 12.53% (EVs^{ABPC}; Figure 4F–G). Additionally, the CD86⁺/F4/80⁺ polarization ratio (M1/M0) was 73.25% after treatment with LPS + IFN- γ . Following treatment with EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC}, the proportion of M1 cells was reduced to 32.75%, 30.32%, and 27.74%, respectively (Figure S5D). These findings further confirmed that EVs^{ABPC} possessed a robust capability to switch from M1 to M2. To investigate the possible mechanism by which EVs^{ABPC} repopulated macrophages, we assessed the relative levels of proteins involved in the inflammatory Nuclear Factor kappa-light-chain-enhancer of activated B cell (NF- κ B) signaling pathway, which is critical for regulating the M1/M2 switch.⁴⁶ We found that EVs^{ABPC} inhibited the NF- κ B pathway, as evidenced by the decreased expression of phosphorylated

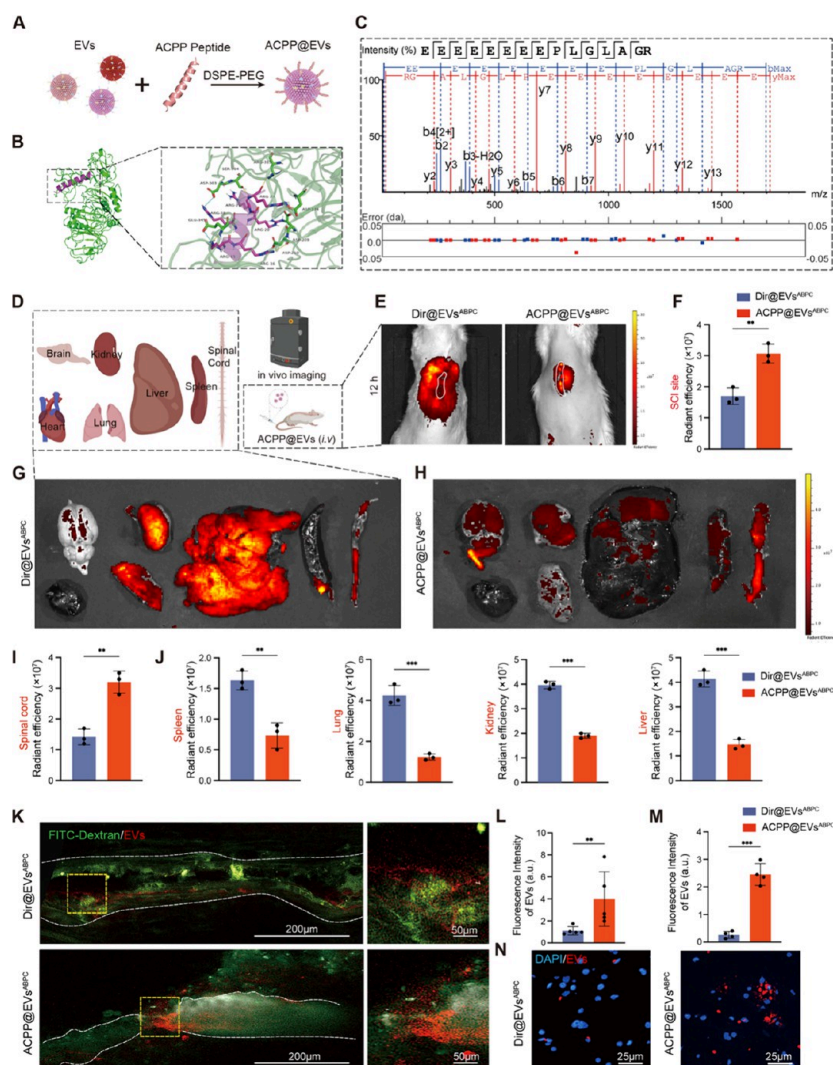


Figure 5. ACPP-modified EVs^{ABPC} enhanced targeted delivery to SCI sites. (A) Schematic diagram illustrating the modification of EVs with ACPPs to form ACPP@EVs. (B) Docking simulation between MMP2 and ACPP peptides showing specific interactions, including hydrogen bonds and salt bridges, with key amino acid residues highlighted in purple. (C) Mass spectrometry analysis confirming the peptide sequence (EEEEEEEEEEPLGLAGR) in ACPP@EVs^{ABPC}, as indicated by the DIA spectrum with fragment ions (red, y ion; blue, b ion) and an error plot showing the measurement accuracy. (D) ACPP@EVs labeled with a red fluorescent protein (RFP) were administered to SCI rats via tail vein injection, and their distribution was monitored 12 h postinjection by *in vivo* and *ex vivo* imaging. (E) Representative *in vivo* images showing the distribution of Dir@EVs^{ABPC} and ACPP@EVs^{ABPC} at the injury site 12 h postinjection. (F) Quantification of radiant efficiency at the injury site ($n = 3$). (G–H) *Ex vivo* imaging of the major organs showing the distribution of Dir@EVs^{ABPC} (G) and ACPP@EVs^{ABPC} (H). (I–J) Quantification of radiant efficiency in the spinal cord (I), spleen, lungs, kidneys, and liver (J) ($n = 3$). (K) Two-photon imaging of the dynamics of EVs from the vasculature to the spinal cord 4 h postinjection, with blood vessels (green) and EVs (red). Scale bar (left), 200 μm ; Scale bar (right), 50 μm . (L) Quantification of fluorescence intensity of EVs in the spinal cord, as visualized by two-photon imaging ($n = 5$). (M) Quantification of the fluorescence intensity of Dir@EVs^{ABPC} and ACPP@EVs^{ABPC} in spinal cord histology ($n = 4$). (N) Representative images of EV distribution in the spinal cord, with DAPI (blue) staining nuclei and Dir@EVs and ACPP@EVs (red). Scale bar, 25 μm . (F, I, J, L, and M) All statistical data are represented as mean $\pm$ SD. Statistical significance between groups was determined using an unpaired Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns: not significant. a.u.: arbitrary unit.

IkB α (p-IkB α) and phosphorylated P65 (p-P65). Collectively, these results demonstrate that EVs^{ABPC} inhibited M1 macrophages and promoted M2 macrophages, probably by regulating the NF- κ B pathway (Figure 4H).

Next, we investigated the immunomodulatory ability of EVs^{ABPC} *in vivo*. Fourteen days following treatment with EVs, the abundance of Arg1⁺ cells was the highest in the EVs^{ABPC} group, followed by the EVs^{NSC} and EVs^{BMSC} groups, and was lowest in the vehicle group. The opposite trend was observed for iNOS⁺ intensity, with the most significant value in the vehicle group and the lowest value in the EVs^{ABPC} group

(Figure 4I–J). These findings confirmed the ability of EVs^{ABPC} to switch M1 macrophages to the M2 phenotype *in vivo*. In addition, EVs treatment significantly attenuated the upregulation of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, in the focal areas after SCI, with EVs^{ABPC} showing the most pronounced inhibition of proinflammatory cytokines (Figure 4K). Thus, EVs^{ABPC} possessed a robust ability to ameliorate inflammatory responses and switch to the M1/M2 transition, which was beneficial for cell survival and tissue regeneration.

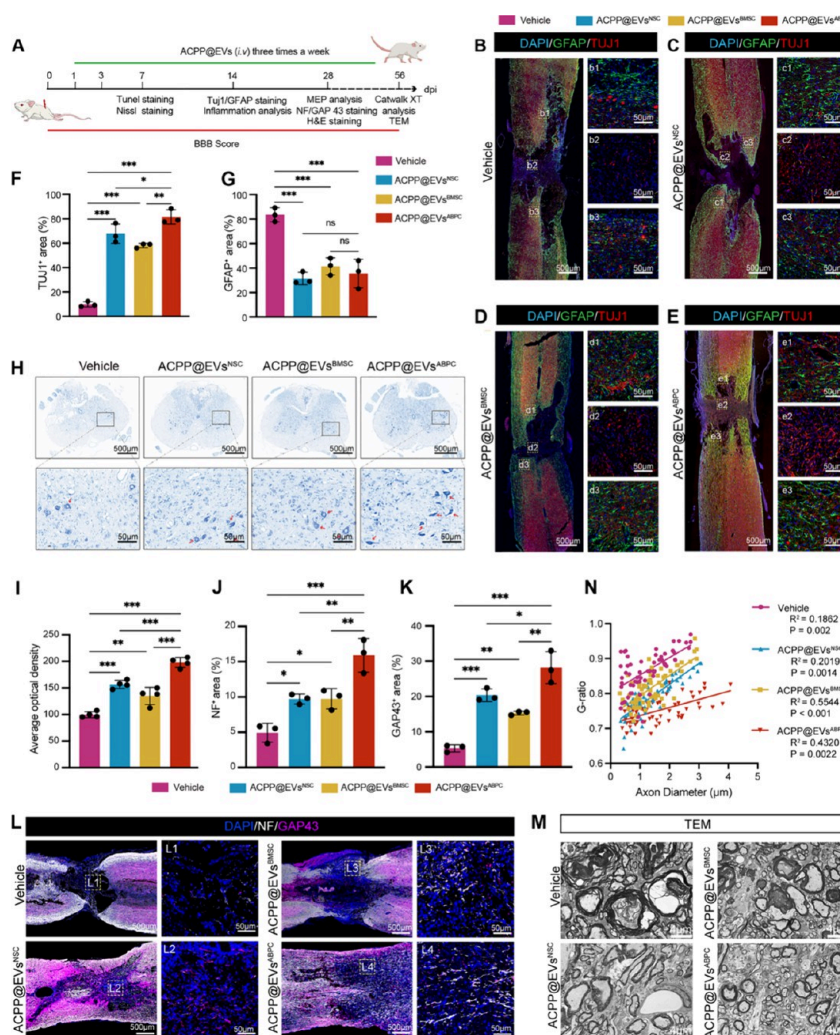


Figure 6. ACPP@EVs^{ABPC} promoted neural regeneration and reduce neuronal apoptosis post-SCI *in vivo*. (A) Schedule of SCI rats treated with ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, and ACPP@EVs^{ABPC}. Treatment included intravenous injections (*i.v.*) three times weekly, followed by assessments at various time points to evaluate neural regeneration and motor function recovery. (B–E) Representative immunofluorescence images of sagittal sections of the injured spinal cord stained for GFAP (green) and TUJ1 (red) to visualize astrocytes and newborn neurons at 14 dpi treated with ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, and ACPP@EVs^{ABPC}, respectively. Scale bar, 500 μ m. Insets show higher-magnification images of the boxed areas (b1–b3, c1–c3, d1–d3, e1–e3). (F–G) Quantification of the areas of TUJ1⁺ neurons (F) and GFAP⁺ astrocytes (G) in the lesion site ($n = 3$). (H–I) Representative Nissl staining images and quantification of the average optical density of neurons at 7 dpi ($n = 5$). Scale bar, 500 μ m. Insets show higher-magnification images of the boxed areas. Scale bar, 50 μ m. (J–K) Quantification of the areas of NF⁺ axons and GAP43⁺ axons at 28 dpi ($n = 3$). (L) Representative immunofluorescence images of sagittal sections of the injured spinal cord stained for NF (white) and GAP43 (purple) to visualize nerve fibers and regenerating axons. Scale bar, 500 μ m. Insets show higher-magnification images of the boxed areas (L1–L4). Scale bar, 50 μ m. (M–N) Representative transmission electron micrographs (M) and scatterplots (N) comparing the myelin thickness and the correlation and linear regression between axon diameter and G-ratio in the vehicle, ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, and ACPP@EVs^{ABPC} treatment groups. Axons were selected from 4 rats per group for measurement ($n = 60$ measured axons for each group). Scale bars, 4 μ m. Correlation analyses were performed using Pearson's correlation. (F, G, I, J, and K) All statistical data are represented as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA with Bonferroni's posthoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns: not significant.

Delivery System for EVs to Target SCI Lesions. Current therapeutic applications of EVs involve nontissue-specific targeting, which results in their nonselective distribution across diverse tissues upon systematic administration.⁴⁷ To address this issue, the surface of EVs was modified with activatable cell-penetrating peptides (ACPPs), which comprised a sequence of polyanionic inhibitory domain, polycationic CPP, and peptide linker matrix that exhibited sensitivity to metalloproteinases (MMPs; Figure 5A).^{48,49} In SCI lesions rich in MMPs, the linker undergoes selective cleavage, causing the dissociation of the inhibitory acidic peptide based on its off-rate, thereby

exposing the cationic CPP for the delivery of EVs into adjacent cells.⁴⁹ Docking analysis revealed specific interactions between MMP2 and ACPP peptides, including multiple hydrogen bonds and salt bridges (Figure 5B). The close-up view highlights critical amino acid residues such as GLU-33, ASN-45, and ARG-67 (colored purple), alongside key interactions like GLU-345 with ARG-15 and ASP-210 with ARG-16, which contribute to stabilizing the binding pocket (Figure 5B, Table 1). Subsequently, we performed mass spectrometry on the ACPP-engineered EVs. Figure 5C shows an example of a DIA spectrum containing the target peptide (EEEEEEEEEEPL-

GLAGR) predicted using DeepNovo-DIA. Each panel highlights fragment ions that corroborate the respective peptide: red denotes γ ions, and blue denotes b ions. The error plot matches the experimentally obtained and theoretical mass peaks, indicating high measurement accuracy (within ± 0.05 Da). Collectively, these results confirmed the peptide sequence and successful construction of ACPP@EVs.

We further labeled ACPP@EVs^{ABPC} with a red fluorescent protein (RFP) to examine its *in vivo* distribution, using Dir-labeled EVs as controls. EVs were then administered to SCI rats via tail vein injection, and their distribution was monitored by *in vivo* imaging of small animals 12 h postinjection (Figure 5D). The fluorescence signals in rats injected with Dir@EVs^{ABPC} were predominantly distributed outside the SCI site. In contrast, in the ACPP@EVs^{ABPC} group, the fluorescence signals were mainly localized at the site of SCI, which was 1.8 times greater than that in the Dir@EVs^{ABPC} group (Figure 5E–F). The major organs of rats were dissected and arranged accordingly, as shown in Figure 5D. In the ACPP@EVs^{ABPC} group, a notable increase in fluorescence signals was observed in the spinal cord, measuring 2.3-fold higher compared to that in the Dir@EVs^{ABPC} group (Figure 5G–I). In contrast, the kidneys, liver, spleen, and lungs showed a decrease in fluorescence to approximately 47.6, 35.7, 45.5, and 29.4% of the levels observed in the Dir@EVs^{ABPC} group, respectively (Figure 5J). These findings indicated that engineering EVs^{ABPC} with ACPP increased their targeting of the spinal cord after injury. Figure S6A–C shows the *ex vivo* distribution of ACPP-loaded EVs^{NSC} and EVs^{BMSC}.

The transport of EVs from the vasculature to the adjacent spinal cord was further visualized using two-photon imaging, with blood vessels marked with FITC (green) and EVs labeled with RFP or Dir (red). Four hours postinjection, high-intensity red fluorescence signals were observed in the infarct zone of spinal cord lesions in the ACPP@EVs^{ABPC} group (Figure 5K). The fluorescence intensity of EVs in the ACPP@EVs^{ABPC} group was nearly 3.6-fold higher than that in the Dir@EVs^{ABPC} group (Figure 5L). Similarly, a significantly higher intensity of red fluorescence was confirmed in the ACPP@EVs^{ABPC} group by histological analysis of local spinal cord lesions, which was 9.4-fold higher than that in the Dir@EVs^{ABPC} group (Figure 5M–N). Thus, using a combination of *in vivo* imaging and *ex vivo* two-photon imaging techniques, we provide direct evidence that ACPP@EVs^{ABPC} selectively accumulates at the SCI sites.

To evaluate the anti-inflammatory properties of ACPP further, we measured the expression levels of proinflammatory cytokines TNF- α and IL-1 β in LPS + IFN- γ pretreated macrophages across three groups: vehicle control (LPS + IFN- γ alone), unmodified EVs^{ABPC}, and ACPP@EVs^{ABPC}. Both the unmodified EVs^{ABPC} and ACPP@EVs^{ABPC} groups significantly reduced TNF- α and IL-6 levels compared to the vehicle control group (Figure S6D). However, no significant difference was detected between the unmodified EVs^{ABPC} and ACPP@EVs^{ABPC} groups, suggesting that the observed anti-inflammatory effect is primarily due to the inherent properties of EVs^{ABPC}.

EVs^{ABPC} Facilitated Neural Network Reconstruction after SCI. Next, we examined the potential therapeutic effects of ACPP@EVs^{ABPC} on neural regeneration in the rat traumatic SCI model (Figure 6A and Figure S7A). After SCI, the rats were administered different ACPP@EVs three times weekly via tail vein injection until sacrifice, after which they were

successfully transferred to the spinal cord lesions *in vivo*. Fourteen days postinjury (dpi), sagittal sections of the injured spinal cord were stained with GFAP to visualize astrocytes and with TUJ1 to mark newborn neurons. In the vehicle group, there was a restricted presence of TUJ1⁺ cells ($9.85 \pm 2.17\%$) observed from the rostral to the caudal of the lesion site, especially the epicenter. This was accompanied by overactivity and accumulation of astrocytes (Figure 6B). In contrast, TUJ1 positive cells were more evenly distributed within the lesion site in the ACPP@EV-treated groups (Figure 6C–E). Among the three types of EVs, ACPP@EVs^{ABPC} exhibited the greatest neuroprotective effect, with $81.59 \pm 5.82\%$ of neurons, which was 1.2 times the proportion found in the ACPP@EVs^{NSC} group ($67.38 \pm 7.92\%$) and more than 1.4 times that in the ACPP@EVs^{BMSC} group ($58.07 \pm 1.80\%$; Figure 6F). Additionally, ACPP@EVs^{ABPC} was significantly more effective than the vehicle in limiting the overactivity and accumulation of astrocytes during secondary injury (Figure 6G). At 7 dpi, we found a significantly higher density of neurons in the ACPP@EVs^{ABPC} group than in the other groups via Nissl staining, which represented a 1.9-fold higher density in the vehicle group, 1.2-fold higher density in the ACPP@EVs^{NSC} group, and 1.5-fold higher density in the ACPP@EVs^{BMSC} group (Figure 6H–I). These findings suggest that EVs^{ABPC} have the ability to optimize the proportion of neurons and astrocytes for the promotion of the spinal cord and have a greater neuroprotective effect than EVs from NSCs and BMSCs.

The primary cause of functional impairments after SCI is the depletion of mature neurons, directly caused by the initiation of programmed cell death in neural cells.⁵⁰ Thus, we examined the antiapoptotic effects of ACPP@EVs^{ABPC} *in vivo* using TUNEL staining (Figure S7B–C). At 7 dpi, the lowest apoptosis in neurons was found in the ACPP@EVs^{ABPC} ($53.7 \pm 3.2\%$) group, followed by the ACPP@EVs^{BMSC} ($71.1 \pm 2.4\%$) and ACPP@EVs^{NSC} ($70.7 \pm 3.5\%$) groups, and the highest in the vehicle group ($84.3 \pm 1.2\%$). These findings suggest a strong antiapoptotic effect of ACPP@EVs^{ABPC}, which may contribute to greater neuronal preservation and facilitate neural network reconstruction after SCI.

At 28 dpi, we immunostained growth-associated protein-43 (GAP43) to visualize regenerated budding axons and neurofilament 200 (NF) to observe mature nerve fibers. Compared with the other groups, GAP43 positive axons in the EVs^{ABPC} group were arranged in a relatively uniform and oriented pattern (Figure 6J–L), indicating better neural network reconstruction after SCI. Additionally, we detected robust neurite regrowth in the ACPP@EVs^{ABPC} group, with significantly larger areas of GAP43 and NF than those in the other groups (Figure 6J–K). Dotted NF⁺ fibers were found in the vehicle group and were mostly limited to injured areas. In contrast, nerve fibers in the different EV groups were distributed across the lesion regions, with a length more than twice that of the vehicle group. Notably, ACPP@EVs^{ABPC} induced the greatest increase in nerve fiber length, which was 1.3-fold longer than that in the ACPP@EVs^{NSC} group and 1.5-fold longer than that in the ACPP@EVs^{BMSC} group (Figure S8A). This finding suggests that EVs^{ABPC} have a greater ability to promote the regrowth of new nerve fibers than EVs from NSCs and BMSCs.

Demyelination leaves axons exposed, making them prone to atrophy and degeneration, which worsens dysfunction. Promoting myelin repair could be a potential strategy for restoring function after SCI.¹⁴ To explore this, we examined

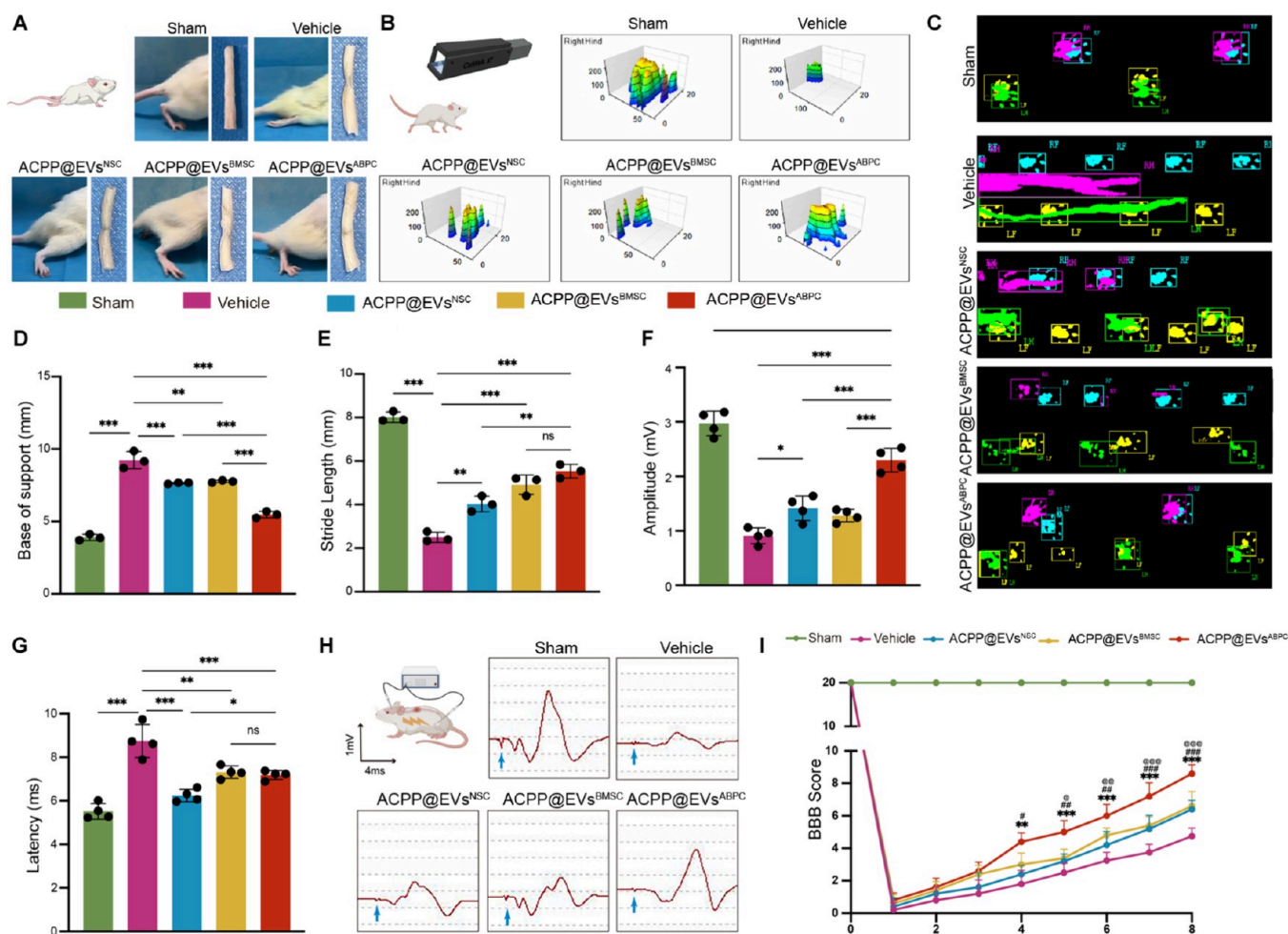


Figure 7. ACPP@EVs^{ABPC} improved motor function recovery post-SCL. (A) Representative images of the right hindlimb of rats during the climbing test showing the effects of treatment with ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, or ACPP@EVs^{ABPC}. (B) Representative 3D stress diagrams of the right hindlimb at 8 wpi. (C) The representative footprints recorded by the Catwalk system (cyan for right front, RF; yellow for left front, LF; green for left hind, LH; and magenta for right hind, RH). (D–E) Quantification of the base of support (D) and stride length (E) in different treatment groups based on footprint patterns ($n = 3$). (F–G) Quantification of CMAP amplitude (F) and latency (G) in the different treatment groups ($n = 4$). (H) Representative MEP waveforms in the different groups. (I) BBB locomotor rating scale of hindlimbs in different groups during the 8-week treatment period ($n = 5$). All statistical data are represented as mean $\pm$ SD (D, E, F, and G). All statistical data are represented as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA with Bonferroni's posthoc test. (I) Statistical differences were determined using two-way ANOVA with Bonferroni's posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the Vehicle group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to ACPP@EVs^{BMSC} group; @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ compared to ACPP@EVs^{NSC} group. ns: not significant.

the effects of EVs^{ABPC} on axonal remyelination. Using transmission electron microscopy, we observed extensive demyelination in the vehicle group, characterized by vacuole-like structures, thinner myelin sheaths, and pronounced delamination (Figure 6M). While there were no significant differences in axonal diameter among groups (Figure S8B), we noted a significant reduction in the G-ratio (axon-to-fiber ratio) in the ACPP@EVs^{ABPC} group (0.75 [95% CI: 0.74–0.76]) compared to that in the other groups (Figure S8C). This suggests that ACPP@EVs^{ABPC} treatment led to a high degree of remyelination. Additionally, although the G-ratio showed a weak correlation with axon diameter, the myelin thickness in rats treated with ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, and ACPP@EVs^{ABPC} was more proportional to axon diameter compared to the vehicle group. Notably, the ACPP@EVs^{ABPC} group exhibited the thickest myelin sheaths, further emphasizing a hypermyelination phenotype (Figure 6N).

Further, we used immunostaining for myelin basic protein (MBP) to visualize remyelinated axons. In contrast to the vehicle group, where few myelinated axons were found at the caudal ends or lesion center, MBP-positive cells and myelinated axons were significantly more abundant in the ACPP@EVs^{ABPC} group after 6 weeks (Figure S8D–E). Together, these findings indicated that ACPP@EVs^{ABPC} treatment accelerated myelination in the central nervous system, suggesting it may be a promising approach for promoting recovery in SCI.

EVs^{ABPC} Improved Motor Function Recovery after SCI.

We assessed the neurological function recovery after 8 weeks of EVs^{ABPC} treatment via comprehensive assessments of behavior, motor function, and neurophysiology. Images of the right claw captured during the climbing test are shown in Figure 7A. The rats in the vehicle group dragged hind limbs in a contracted state two months postsurgery. In contrast, after treatment with different EVs, rats exhibited slow and

coordinated movements of both hind limbs, with the most significant improvement observed in the EVs^{ABPC} group.

Following this, we conducted a thorough quantitative assessment of motor function using the CatWalk system at 8 weeks postinjury (wpi).⁵¹ Gait analysis of SCI rats was limited to forepaw data because of hindlimb paralysis. A typical 3D image showed that the hind toes of EV-treated rats supported their weight, with the most significant improvement observed in the EVs^{ABPC}-treated group (Figure 7B). In addition, the footprints of rats in the vehicle group exhibited a linear pattern, indicating continuous hindlimb dragging. In contrast, EVs^{ABPC} group rats were able to lift their hindlimbs off the ground, exhibiting frequent plantar stepping and occasional forelimb-hindlimb coordination, demonstrating recovery of motor function (Figure 7C). Additionally, ACPP@EVs^{ABPC} significantly decreased the support base in SCI rats while increasing stride length, demonstrating a substantial improvement in locomotor coordination between the fore and hind limbs (Figure 7D–E).

Motor-evoked potential (MEP) testing revealed that compound muscle action potential (CMAP) signals from the right hind limbs in the vehicle group decreased to approximately 31.25% of those in the sham group 8 weeks post-SCI. In contrast, the CMAPs signal amplitude was 2.3 ± 0.2 mV in the ACPP@EVs^{ABPC} group, which was approximately 1.6-fold higher than that in the ACPP@EVs^{NSC} group, 2.1-fold of that in the ACPP@EVs^{BMSC} group, and 2.6-fold of that in the vehicle group (Figure 7F–H). Additionally, the Basso-Beattie-Bresnahan (BBB) assay was utilized to evaluate locomotor function recovery, conducted 1 day prior to injury and again until sacrifice. At 56 dpi, the rats in vehicle group continued to drag their hind limbs. In contrast, we observed the best motor function improvement in the ACPP@EVs^{ABPC} group, whereas rats in the ACPP@EVs^{NSC} and ACPP@EVs^{BMSC} groups displayed plantar placement with some degree of support; however, their stepping lacked coordination (Figure 7I). Collectively, these results demonstrate that ACPP@EVs^{ABPC} is superior to ACPP@EVs^{NSC} and ACPP@EVs^{BMSC} in enhancing neurological function after SCI.

We also assessed the biomedical safety of ACPP@EVs^{ABPC}. As shown in Figure S9A, histological analysis of major organs was conducted at 28 dpi. Consistent with expectations, Hematoxylin and Eosin (H&E) staining revealed no significant tissue damage in cardiomyocytes, hepatic cells, splenic tissues, alveolar cells, or renal glomeruli (Figure S9A). Additionally, we performed a quantitative analysis of inflammatory cells (including monocytes and lymphocytes), which showed that the number of inflammatory cells remained within the normal range (Figure S9B).⁵² To further verify the biosafety of ACPP@EVs^{ABPC}, we evaluated a complete blood count (Figure S9C), along with ALT, AST, BUN, and cholesterol levels (Figure S9D) at 28 dpi, assessing liver function, kidney function, lipid metabolism, and immune status. The results showed no significant abnormalities across different treatment groups, indicating that ACPP@EVs^{ABPC} had no adverse effects on major physiological functions.

DISCUSSION

This study identified ABPCs as a viable source of EVs that contain an abundance of unique pro-regenerative factors, including proneurogenic factors (RPS2, ACTG1, and RHOA) and immunomodulatory factors (Timp1, B2M, and VWF). In this study, we found that EVs^{ABPC} facilitated the proliferation

of NSCs and promoted the extension of axons in differentiated neurons. In addition, EVs^{ABPC} showed a stronger neuro-protective effect in neurons than EVs from NSCs and BMSCs by enhancing neurite outgrowth and mitigating apoptosis. Moreover, EVs^{ABPC} exhibited strong immunomodulatory ability by switching the activation of macrophages from the inflammatory M1 phenotype toward the reparative M2 phenotype. In the therapeutic context, EVs^{ABPC} achieved better outcomes at both the histological and functional levels in rats with SCI than the most extensively studied EVs (EVs^{NSC} and EVs^{BMSC}). These findings suggest that ABPCs are a novel and practical source of EVs that contain a wealth of systemic pro-regenerative factors and have considerable translational value in the treatment of SCI.

The therapeutic potential of NSCs and BMSCs in regenerative medicine is widely recognized.^{53,54} Numerous studies have increasingly noted that the advantageous effects of stem cells are primarily attributed to their paracrine activity rather than the persistent engraftment of transplanted stem cells.¹⁵ Over the past decade, stem cell-derived EVs have been recognized as crucial mediators of stem cell paracrine action.¹² In addition, both preclinical and clinical studies indicate that EVs derived from stem cells can provide comparable therapeutic benefits to those of the donor cells themselves while circumventing the biosafety concerns associated with stem cell therapy.²⁹ Therefore, to avoid ethical and safety concerns regarding the direct application of xenogeneic and allogenic cells, EVs from different stem cells were used as they lack the ability to self-replicate, thereby addressing concerns about the potential risk of tumor formation following stem cell transplantation.⁵⁵ In this study, we found the potent ability of EVs^{ABPC} to promote nerve regeneration and modulate inflammatory responses, which was stronger than that of EVs from NSCs and BMSCs. The benefits of EVs^{ABPC} are likely due to their unique stemness and renewability of ABPCs. ABPCs can be expanded *in vitro* for at least 50 passages without significant senescence, whereas MSCs exhibit signs of senescence after 10–15 rounds of *in vitro* culture.^{22,25} These characteristics make ABPCs ideal for producing stable phenotypic EVs in large quantities to support EV-based therapies.

The mechanisms through which ABPCs enable regeneration are complex and diverse. An intriguing observation was the rapid growth of nerve fibers toward the antler tip, where ABPCs reside.^{22,32} Therefore, chemotaxis cues within ABPCs are likely to play a pivotal role in guiding the directional growth of nerve fibers, of which EVs may be a major mediator for their communication. In addition, the natural process of antler shedding in deer, which occurs without inflammation, highlights the intrinsic anti-inflammatory properties of ABPCs, which are critical for tissue healing and regeneration. Additionally, ABPCs exhibit high expression levels of genes associated with many pathways, notably the Hippo and Wnt signaling pathways. These pathways are fundamental for enhancing the regenerative functions of ABPCs and supporting cellular processes that are crucial for recovery. The Hippo signaling pathway is a conserved mechanism crucial for regulating cell proliferation and apoptosis.⁴² YAP functions as a mechanically activated downstream effector of this pathway.⁵⁶ In addition, the Wnt signaling pathway promotes the survival and regeneration of neural cells by activating downstream effectors such as β -catenin.^{41,57} The careful modulation of these pathways by ABPCs underlines their

potential in therapeutic applications, bridging critical gaps in current regenerative medicine approaches and offering novel avenues for treatment.

EVs^{ABPC} offer several advantages in translational research. First, EVs^{ABPC} carry unique regenerative signals closely associated with the distinctive regenerative properties of ABPCs. Unlike BMSCs and NSCs, ABPCs can be consistently sourced from the annual regeneration cycle of sika deer antlers, resulting in higher EV yields and greater potential for neural regeneration.²² Additionally, in our study, EVs^{ABPC} were modified with activated cell-penetrating peptides (ACPP) to enhance targeted delivery to the injury site.⁴⁹ Previous studies have shown that unmodified EVs are rapidly cleared in vivo and exhibit low retention rates. In contrast, the ACPP modification in our study significantly improved the local retention and therapeutic efficacy of EVs^{ABPC}, demonstrating superior neural regeneration and motor function recovery in the SCI model. Finally, the utilization of EVs^{ABPC} presents a viable strategy to overcome the limitations of stem cell therapies, including low retention and survival rates due to immune rejection. Furthermore, EVs are stable during long-term frozen storage or storage at room temperature after lyophilization, which is important for translational purposes.^{58,59}

With the development of novel treatments, several concerns must be addressed before advancing current findings for clinical applications. First, a limitation of this study is the selection of the SCI model. Although the clip compression model offers certain advantages in simulating chronic compression and ischemic environments, it does not fully capture the complex pathology of human SCI. To further assess the clinical translational potential of our findings, future studies could incorporate multiple SCI models, such as those created using the IH Impactor or MASCIS Impactor, which may better simulate the complex pathology resulting from acute contusive injuries.^{60,61} Furthermore, to increase clinical relevance, these findings should be replicated across different animal models. Conducting experiments in rats, mice, pigs, and even nonhuman primates would provide additional validation of EVs^{ABPC}'s safety and efficacy to enhance understanding of its therapeutic potential. Second, although EVs are safe for use in rodents and nonhuman primates because of their cell-free properties, a comprehensive study, including long-term safety monitoring (such as 3 or 6 months), is necessary to exclude any potential pro-tumorigenic effects.⁶² An additional safety concern regarding EVs from different species is possible adverse immunological reactions, although this phenomenon was not observed in the current study.⁶² Third, given the profound effect of EVs^{ABPC} on NSC proliferation and neurite outgrowth, a tissue-specific targeting strategy for EVs may allow neuroprotective treatment in specific tissues. In addition, while the current extraction techniques have provided promising results, future studies could incorporate advanced techniques for more precise and scalable EV isolation, especially microfluidic technologies. Incorporating these innovations in EVs extraction can minimize contamination risks and reduce time and labor, addressing scalability challenges associated with traditional methods.⁶³ Finally, EVs^{ABPC}-associated factors critical for dictating function need to be thoroughly assessed, which may reveal new factors that do not exist in other stem cells and potentially provide alternative translational avenues for specific tissue injury and repair.

CONCLUSIONS

ABPCs are a unique type of mesenchymal stem cell that drives complete organ regeneration in mammals annually. EVs^{ABPC} contain pro-regenerative signals that differ from those in EVs derived from other stem cells. EVs^{ABPC} boost neural stem cell proliferation, activate neuronal regenerative potential, modulate immune response, and switch the M1/M2 polarization of macrophages. Engineered EVs^{ABPC} delivered intravenously significantly improve axonal growth, reduce astrogliosis, and greatly enhance motor functional recovery after SCI, outperforming EVs from other stem cell sources. These findings suggest that ABPCs are a practical source of EVs containing pro-regenerative factors and offer a promising therapeutic strategy for SCI repair.

EXPERIMENTAL SECTION

Primary Extraction and Culture of Antler Blastema Progenitor Cells (ABPCs). ABPCs were isolated according to a previously established protocol detailed by Qin *et al.*, published in *Science*.²² Briefly, the hard antlers of a two-year-old male sika deer were used to extract ABPCs. Five days after the antlers were cast, cells within the blastema were sorted using flow cytometry to identify CX43⁺FGFR2⁺ cells as ABPCs. Single-cell suspensions were prepared in Dulbecco's modified Eagle Medium (DMEM; Gibco, 11965092) with 1% penicillin-streptomycin (Gibco, 15140122), 0.1% Mycoplasma Removal Agent, and fetal bovine serum (FBS; Gibco, 10099141C). Subsequently, these cells were plated in 10 cm culture dishes at a density of 2×10^5 cells/cm². After 24 h, nonadherent cells were removed, and adherent cells were cultured. Upon reaching 80–90% confluence, the cells were harvested and reseeded at the same density for further expansion. ABPCs at passages 6–8 (P6–P8) were used in the experiments. This adjusted protocol ensures the effective isolation and maintenance of ABPCs with strong regenerative capabilities that are ready for further experimental use.

Primary Extraction and Culture of Neural Stem Cells. NSCs were extracted from the cerebral cortex of E14 SD rat embryos. Under sterile conditions, the cerebral cortex was carefully dissected from the embryos and mechanically dissociated into a single-cell suspension using fine forceps and gentle pipetting. The resulting cell suspension was cultured in Neurobasal medium (Gibco, Carlsbad, CA, USA) supplemented with 20 μ L/mL B27 (Gibco, Carlsbad, CA, USA), 20 ng/mL epidermal growth factor (EGF, PeproTech), 20 ng/mL basic fibroblast growth factor (bFGF, Invitrogen, Carlsbad, CA, USA), and 1% penicillin-streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂, and the culture medium was changed every 2–3 d to ensure optimal growth conditions. When the cells aggregated into neurospheres and reached 80–90% confluence (P0), they were passaged using TrypLE Express (Thermo Fisher Scientific). Throughout the culture period, cell morphology and proliferation were regularly observed under a microscope to monitor cell health and growth. The presence of NSCs was confirmed using immunofluorescence staining for specific markers such as Nestin and SOX2, ensuring the effective isolation and culture of NSCs for subsequent experimental procedures. The NSCs were divided into three groups: vehicle Group, NSCs treated with the standard culture medium; EVs^{BMSC} Group, NSCs treated with EVs^{BMSC} (2×10^6 particles/mL) for 24 h; and EVs^{ABPC} Group, NSCs treated with EVs^{ABPC} (2×10^6 particles/mL) for 24 h.

Primary Extraction and Culture of DRG Neurons and Explants. SD rat pups aged 1–3 days were used for this procedure. The heads of the pups were removed, and the skin and bones along the spine and thoracic and cervical vertebrae were incised with microscissors to fully expose the DRG on both sides of the spinal canal. Each small bulbous dorsal root ganglion was removed using fine ophthalmic forceps. The outer membrane surrounding the DRG neurons was carefully peeled off, and the ganglia were cut into small pieces.⁶⁴ Collagenase type IV and 0.25% trypsin were added to the dish to digest DRG neurons, and digestion was terminated after 1 h

by adding 4 mL of DF-12 medium containing FBS. The mixture was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in Neurobasal complete medium, filtered through a 100 μ m cell strainer, and inoculated onto cell culture plates that had been coated with matrix gel overnight. The same procedure was followed for DRG explants, but the ganglia were not cut into pieces before digestion, and the digestion time was reduced to 5 min. The DRG neurons and explants were treated with PBS, EVs^{NSC}, EVs^{BMSC}, or EVs^{ABPC} (2×10^6 particles/mL) for 48 h. The effects of the different treatments on DRG neurons and explant health and growth were observed and analyzed. Sholl analysis was conducted using ImageJ with the Sholl Analysis plugin, where concentric circles were drawn around the soma, and the number of dendritic intersections at each radius was quantified. Statistical analyses were conducted to compare the differences across groups.

Culture of RAW 264.7 Cells. RAW 264.7 cells (Procell, Wuhan, China, Cat# CL-0190) were divided into several groups. In the vehicle Group, the medium was replaced with fresh growth medium, with no additional treatment applied. In the LPS + IFN- γ group, RAW 264.7 macrophages were incubated with LPS (100 ng/mL, #L3129, Sigma-Aldrich) and IFN- γ (20 ng/mL, #L17001, Sigma-Aldrich) for 24 h, followed by a medium change to the normal growth medium. In the LPS + IFN- γ + EVs^{NSC} group, macrophages were treated with LPS and IFN- γ for 24 h, followed by a medium change to normal growth medium and the addition of EVs^{NSC} (2×10^6 particles/mL) for 24 h. In the LPS + IFN- γ + EVs^{BMSC} group, macrophages were treated with LPS and IFN- γ for 24 h, followed by a medium change and the addition of EVs^{BMSC} (2×10^6 particles/mL) for 24 h. In the LPS + IFN- γ + EVs^{ABPC} group, macrophages were treated with LPS and IFN- γ for 24 h, followed by a medium change and the addition of EVs^{ABPC} (2×10^6 particles/mL) for 24 h. All groups were incubated under the same conditions. After the treatment period, the cells were further processed to evaluate the effects of EVs on macrophage activation and polarization.

Culture of VSC 4.1 Motoneurons. VSC 4.1 neurons (EditGene #EDWT0066) were grown in a fully humidified incubator at 37 °C and 5% CO₂ in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. In the LPS group, VSC 4.1 neurons were incubated with LPS (1000 ng/mL, #L3129, Sigma-Aldrich) for 24 h. For the LPS + EVs^{NSC} group, VSC 4.1 motoneurons were incubated with LPS for 24 h, followed by a medium change to normal growth medium and the addition of EVs^{NSC} (2×10^6 particles/mL) for 24 h. In the LPS + EVs^{BMSC} group, VSC 4.1 motoneurons were incubated with LPS for 24 h, followed by a medium change to normal growth medium and the addition of EVs^{BMSC} (2×10^6 particles/mL) for 24 h. In the LPS + EVs^{ABPC} group, VSC 4.1 motoneurons were incubated with LPS for 24 h, followed by a medium change to normal growth medium and the addition of EVs^{ABPC} (2×10^6 particles/mL) for 24 h.

EVs Isolation and Characterization. EVs with a diameter greater than 100 nm were isolated and purified using gradient centrifugation, following a previously described protocol. We first cultured ABPCs using a serum-free medium to avoid contamination with extracellular vesicles. Cell viability was assessed using the CCK-8 assay, ensuring that only culture supernatants from cells with over 90% viability were used for EV collection and purification. The collected supernatant was initially centrifuged at $750 \times g$ for 20 min to remove cells, followed by a second centrifugation at $2,000 \times g$ for 30 min to further eliminate cell debris and apoptotic bodies. The supernatant was then centrifuged at $16,000 \times g$ for 70 min to precipitate the EV particles. The obtained pellet was resuspended in prechilled PBS and centrifuged again at $16,000 \times g$ for 70 min to ensure purity. All centrifugation steps were performed at 4 °C to maintain EV integrity. The final EV pellet was resuspended in 100 μ L of prechilled PBS and stored at -80 °C for subsequent analysis. EVs were then characterized using transmission electron microscopy (TEM; TECNAI Spirit, FEI, USA), nanoparticle tracking analysis (NTA; ZetaView, Particle Metrix, Germany), and Western blotting. EVs were quantified using both NTA and bicinchoninic acid (BCA) analysis (Beyotime Biotechnology, Shanghai, China). The concentration and size distribution of the EVs were assessed using NTA,

while BCA analysis was employed for quantification. For the Western blotting process to identify EV markers, 2 μ g of lysates from NSCs, BMSCs, and ABPCs, and 2 μ g of EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC}, were loaded into each lane. For the experiments *in vitro*, we used extracellular vesicles (EVs) at a concentration of 2×10^6 particles/mL, corresponding to a protein concentration of 50–70 μ g/mL as determined by the BCA assay. For *in vivo* experiments, the administered dosage was 1×10^9 particles per rat, with the protein concentration ranging from 500 to 700 μ g/mL.

Single Particle Interferometric Reflectance Imaging Sensor with ExoView. The characterization of EVs was performed using the ExoView platform (ExoView, Boston, MA, USA), which allows for a detailed analysis of EV size, concentration, and surface marker expression to further validate the purity of our EV samples.⁶⁵ First, the isolated EVs were resuspended in precooled PBS at a concentration of 1×10^6 particles/mL, which is suitable for ExoView analysis. The prepared EV samples were then loaded onto ExoView chips (EV-TM-FLEX-CAR), which are specifically designed to capture EVs based on their surface markers. The ExoView platform uses fluorescently labeled antibodies to specifically detect surface markers on the EVs, providing detailed information on the presence and abundance of these markers. The ExoView system captures high-resolution images of the EVs, enabling the precise measurement of their size and concentration. The data obtained from the ExoView system were analyzed using its software that generated comprehensive reports on the size distribution, concentration, and surface marker profiles of the EVs.

Liquid Chromatography–Mass Spectrometry (LC–MS/MS). Protein (100 μ g) from each sample was digested using a filter-aided sample preparation (FASP) method for proteomic analysis. The resulting peptides were desalted using C18 spin columns (Thermo Fisher Scientific) and dried via vacuum centrifugation. The mass spectrometer was operated in data-dependent acquisition mode by selecting the top 20 most abundant precursor ions for HCD fragmentation.

Proteomics Analysis. Raw MS data were processed using the MaxQuant software (version 1.6.1.0). The MS/MS spectra were searched against the UniProt human protein database. Carbamidomethylation of cysteine was designated as a fixed modification, while oxidation of methionine and acetylation of protein N-termini were set as variable modifications. A maximum of two missed cleavages was allowed. Peptide and protein identifications were filtered using a 1% false discovery rate (FDR). Label-free quantification (LFQ) was performed using the MaxLFQ algorithm. Quantified proteins were analyzed using Perseus software (version 1.6.2.3). Only proteins identified in at least two out of the three biological replicates with LFQ intensity values were considered. Regarding the analysis methods for differentially expressed proteins, we employed the limma package in R (version 4.3.2) to perform the analysis of gene expression. Initially, standard preprocessing steps, including quality control, normalization, and filtering, were conducted to ensure data integrity. A linear model was then fitted to log₂-transformed expression values using the lmFit function to assess the effects of experimental conditions. Following this, the eBayes function was applied to compute moderated t-statistics, log-fold changes, and associated p-values for each protein. To identify differentially expressed proteins, thresholds of absolute log-fold change ($|\log_2 \text{FC}| > 1.5$) and $P < 0.05$ were used. Functional enrichment analysis was performed using Metascape (<https://metascape.org>) ($P < 0.05$).

Fluorescent Ubiquitination-Based Cell Cycle Indicator (FUCCI) System. A lentiviral vector containing an A FUCCI reporter gene (EFLA-mKO2-T2A-mAzami-Green) was developed by Genechem, China.³⁶ This vector is designed to be selectively expressed at specific cell cycle stages. The utilization of reporter genes with different fluorescent colors allows for real-time imaging of the various phases of the cell cycle, where red, yellow, and green fluorescence correspond to cells in the G₁, G₁/S transition, and S/G₂-M phases, respectively. The specific synthesis method was as follows: the target gene was PCR-amplified, followed by digestion of the vector with PacI and EcoRI. The PCR product was then purified.

Next, the purified PCR fragment was ligated into the digested vector to obtain the recombinant plasmid. This recombinant plasmid was subsequently transfected into suitable packaging cells along with helper plasmids to produce the lentivirus. Finally, we collected the viral supernatant, concentrated it, and used it to infect and label the NSCs. Subsequently, the labeled NSCs were treated with PBS, EVs^{BMSC} (2×10^6 particles/mL), and EVs^{ABPC} (2×10^6 particles/mL) for 24 h. Following treatment, the cells were observed and analyzed statistically analysis using the High Content Imaging System (ImagePress Micro, Molecular Devices).

Preparation of ACPP@EVs. First, a Tris-HCl buffer solution was prepared by dissolving 0.44 g of Tris-HCl in 45 mL of deionized water, and the pH was adjusted to 6.8–7.4 using a 1% ammonia solution. Next, 5 mg of DSPE was dissolved in 200 μ L of anhydrous ethanol. The peptide, EEEEEEEE(E8)-6-aminohexanoyl-PLGLAG-RRRRRRRR(R8),⁴⁹ was dissolved in 1 mL of Tris-HCl buffer and added to the DSPE solution, followed by rinsing the peptide container twice with Tris-HCl buffer and adding the rinses to achieve a total volume of 3.2 mL. Then, 1.8 mL of Tris-HCl buffer was added to bring the final volume to 5 mL. The reaction mixture was protected from light by wrapping the tube in aluminum foil and mixing overnight on a shaker. Subsequently, EVs^{NSC}, EVs^{BMSC}, and EVs^{ABPC} (1×10^{11} particles/mL) were added to the reaction mixture and incubated for an additional period to allow conjugation. The solution was transferred to a dialysis membrane and dialyzed against 2 L of deionized water with an appropriate amount of anhydrous ethanol while stirring overnight. The water was changed every 12 h to ensure thorough purification. Finally, the number of purified EV particles number was measured using NTA at a concentration of approximately 1×10^{10} particles/mL.

Validation of Targeted EV Construction Using Mass Spectrometry. We performed a mass spectrometry (MS) analysis. The engineered EVs were lysed, and the proteins were digested with trypsin. The resulting peptides were then purified and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The raw data were processed and analyzed using PEAKS Studio X+ software (Bioinformatics Solutions Inc.) to identify specific marker proteins and peptides that indicated successful targeting and EV construction.⁶⁶

Animals. We used adult female Sprague–Dawley (SD) rats, aged 8–10 weeks and weighing approximately 220–250 g. These rats were obtained from the Animal Experiment Centre of The Fourth Military Medical University, China (License No. IACUC-20241365), and were housed in clean cages at a controlled temperature of 22–25 °C, with a 12-h light/dark cycle and ad libitum access to food and water. All experimental procedures were approved by the Ethics Committee of The Fourth Military Medical University, adhering to the 3Rs principles (Replacement, Reduction, and Refinement) to minimize the number of animals used and reduce suffering. The rats were randomly divided into five experimental groups, with at least five rats in each group: 1) Sham group, which received surgery without SCI induction; 2) Vehicle group, untreated SCI rats; 3) ACPP@EVs^{NSC} group, SCI rats treated with intravenous ACPP@EVs^{NSC} at a dose of 1×10^9 particles per injection three times weekly; 4) ACPP@EVs^{BMSC} group, SCI rats treated with intravenous ACPP@EVs^{BMSC} at a dose of 1×10^9 particles per injection three times weekly; and 5) ACPP@EVs^{ABPC} group, SCI rats treated with intravenous ACPP@EVs^{ABPC} at a dose of 1×10^9 particles per injection three times weekly. Sampling time points were set at 3, 7, 14, 28, and 56 dpi to evaluate the repair outcomes at different stages. At each time point, the BBB score was used to assess motor function recovery. Specific analyses included Nissl staining at 7 dpi to observe neuronal density, GFAP and TUJ1 staining at 14 dpi to evaluate the distribution of astrocytes and newly formed neurons, quantification of nerve fiber length and electrophysiological analysis at 28 dpi, HE staining and blood biochemistry at 28 dpi, and MBP staining and TEM analysis of myelin structure at 56 dpi.

SCI Model. Rats were anesthetized via intraperitoneal injection of sodium pentobarbital and placed in a prone position. The dorsal area was shaved, and the skin was disinfected with iodophor. After

disinfection, the skin, subcutaneous tissue, and paravertebral muscles were carefully dissected to expose the T9 vertebra. The T9 spinous process and vertebral plate were removed to expose the spinal cord without causing injury, using fine forceps. The spinal cord was then compressed vertically using microvascular clips (RWD Life Science, R31005–10) paired with a clip applicator (RWD Life Science, R34001–14) for 5 s. Successful compression was confirmed by observing limb extension and tail twitching. Postcompression, muscles, and skin were sutured using sterile techniques, and cefuroxime was administered intraperitoneally. The rats were allowed to recover at room temperature. Postoperatively, rats in the ACPP@EVs^{NSC}, ACPP@EVs^{BMSC}, and ACPP@EVs^{ABPC} groups received their respective EVs via intravenous injections three times weekly until sacrifice. Manual bladder expression was also performed twice daily until reflex bladder emptying was restored.

Rats were humanely euthanized with an overdose of isoflurane at 3, 7-, 14-, 28-, and 56-days post-SCI. A median thoracoabdominal incision was made, followed by perfusion with 10 mL of cold saline via the left ventricle and then 100 mL of cold 4% paraformaldehyde (Beyotime, Shanghai, China, Cat# P0099–100 mL). The spinal cord segments (0.5 cm) above and below the lesion site were harvested and fixed overnight in 4% paraformaldehyde.

Electrophysiological Analysis. Electrophysiological tests were performed at 28 dpi to evaluate the functional status of sensorimotor signal conduction. The sciatic nerves of the rats were exposed under anesthesia. For motor-evoked potential (MEP) measurements, a stimulating electrode was inserted into the spinal motor cortex (SMC), and a recording electrode was placed into the sciatic nerve. The waveforms, amplitudes, and latencies of the MEPs were recorded and analyzed.

Gait Analysis. The Catwalk XT (Noldus) system was employed to identify neurological gait disorders. One week before modeling, the rats were trained to learn the procedure. Gait analysis was conducted in the fourth week after EV treatment to assess the recovery of motor function. The system recorded the footprints of the rats as they voluntarily crossed a glass plate toward a goal box. For the analysis, a minimum of three successful runs were required for each group. Additionally, based on the measurement data, the Catwalk XT system was used to calculate the BBB score.

Statistical Analysis. Statistical analysis was conducted using GraphPad Prism 9.2.0 software (GraphPad Software, San Diego, CA, USA). We used the Shapiro–Wilk test to assess normality for each group's data distribution. The homogeneity of variances was evaluated using the F-test for two-group comparisons and the Brown–Forsythe test for comparisons involving more than two groups. When data met the assumptions of normality and homogeneity of variances, they were reported as mean $\pm$ standard deviation (SD), and we performed an unpaired *t* test for two-group comparisons or one-way ANOVA followed by Bonferroni's multiple comparisons test for comparisons among multiple groups. For data with two variables (group and time), two-way ANOVA was applied. For data deviating from a normal distribution, we applied the nonparametric Mann–Whitney U test for two-group comparisons and the Kruskal–Wallis test, followed by Dunn's multiple comparisons test for multigroup comparisons. These data were reported as median with interquartile range (IQR). Correlation analyses were performed using Pearson's correlation. Detailed statistical methods are provided in each figure legend. All differences among and between groups were considered statistically significant at *P* < 0.05. Significance levels were defined as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.4c10298>.

Detailed experimental procedures including descriptions of primary extraction and culture of BMSCs, ELISA for cytokine quantification, H&E and Nissl staining

procedures, two-photon microscopy for EV transport dynamics, RNA sequencing analysis, transmission electron microscopy, molecular mechanism analysis, and flow cytometry for macrophage analysis; comparative proteomic analysis of EVs^{ABPC}, EVs^{NSC}, and EVs^{BMSC} (Figure S1); identification of NSCs and analysis of the effects of EVs^{BMSC} and EVs^{ABPC} on NSCs proliferation and differentiation (Figure S2); enhancement of neurite outgrowth and reduction of neuronal apoptosis by EVs^{ABPC} (Figure S3); bioinformatics analysis and validation of key signaling pathways in neurons treated with EVs^{ABPC} (Figure S4); evaluation of inflammatory response and macrophage polarization induced by different EVs treatments (Figure S5); *ex vivo* distribution of ACP@ EVs (Figure S6); evaluation of neuronal apoptosis and treatment effects post SCI (Figure S7); ACP@EVs^{ABPC} promoted neural regeneration and axonal remyelination in SCI (Figure S8); safety evaluation of EVs^{ABPC} treatment on major organs and physiological parameters (Figure S9); and analysis of the protein interaction between MMP2 and ACP peptide segments (Table 1) (PDF)

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Notes

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ABBREVIATIONS

SCI:spinal cord injury; MSC:mesenchymal stem cell; NGF:nerve growth factor; IGF-1:insulin-like growth factor-1; ABPCs:antler blastema progenitor cells; EV:extracellular vesicle; ACP:activated cell-penetrating peptides; TEM:transmission electron microscopy; LC-MS/MS:liquid chromatography–tandem mass spectrometry; PCA:principal component analysis; GO:gene ontology; DEP:differentially expressed protein; Fucci:fluorescent ubiquitination-based cell cycle indicator; LPS:lipopolysaccharide; IFN- γ :interferon-gamma; DEG:differentially expressed gene; GSEA:gene set enrichment analysis; iNOS:inducible nitric oxide synthase; TNF- α :tumor necrosis factor- α ; MBP:myelin basic protein; FACS:fluorescence-activated cell sorting; PBS:phosphate-buffered saline; MEP:metalloproteinase (MMP); Motor-evoked potential; CMAP:compound muscle action potential; BBB:Basso–Beattie–Bresnahan; dpi:days postinjury; BMSC:bone marrow stem cell; NSC:neural stem cell

REFERENCES

- Rees, P. M.; Fowler, C. J.; Maas, C. P. Sexual Function in Men and Women with Neurological Disorders. *Lancet* **2007**, 369 (9560), 512–525.
- Cramer, M. N.; Gagnon, D.; Laitano, O.; Crandall, C. G. Human Temperature Regulation under Heat Stress in Health, Disease, and Injury. *Physiol. Rev.* **2022**, 102 (4), 1907–1989.
- Lembo, T.; Munakata, J.; Mertz, H.; Niazi, N.; Kodner, A.; Nikas, V.; Mayer, E. A. Evidence for the Hypersensitivity of Lumbar Splanchnic Afferents in Irritable Bowel Syndrome. *Gastroenterology* **1994**, 107 (6), 1686–1696.
- DeVivo, M. J. Epidemiology of Traumatic Spinal Cord Injury: Trends and Future Implications. *Spinal Cord* **2012**, 50 (5), 365–372.
- Divanoglou, A.; Westgren, N.; Seiger, Å.; Hulting, C.; Levi, R. Late Mortality During the First Year After Acute Traumatic Spinal Cord Injury: A Prospective, Population-Based Study. *J. Spinal Cord Med.* **2010**, 33 (2), 117–127.
- Furlan, J. C.; Fehlings, M. G. The Impact of Age on Mortality, Impairment, and Disability among Adults with Acute Traumatic Spinal Cord Injury. *J. Neurotrauma* **2009**, 26 (10), 1707–1717.
- Yilmaz, T. Current and Future Medical Therapeutic Strategies for the Functional Repair of Spinal Cord Injury. *World J. Orthop.* **2015**, 6 (1), 42.
- Hu, X.; Xu, W.; Ren, Y.; Wang, Z.; He, X.; Huang, R.; et al. Spinal Cord Injury: Molecular Mechanisms and Therapeutic Interventions. *Signal Transduction Targeted Ther.* **2023**, 8 (1), 245.
- Zholudeva, L. V.; Qiang, L.; Marchenko, V.; Dougherty, K. J.; Sakiyama-Elbert, S. E.; Lane, M. A. The Neuroplastic and Therapeutic Potential of Spinal Interneurons in the Injured Spinal Cord. *Trends Neurosci.* **2018**, 41 (9), 625–639.
- Fischer, I.; Dulin, J. N.; Lane, M. A. Transplanting Neural Progenitor Cells to Restore Connectivity after Spinal Cord Injury. *Nat. Rev. Neurosci.* **2020**, 21 (7), 366–383.
- Liu, H.; Zhang, J.; Xu, X.; Lu, S.; Yang, D.; Xie, C.; et al. SARM1 Promotes Neuroinflammation and Inhibits Neural Regeneration after Spinal Cord Injury through NF- κ B Signaling. *Theranostics* **2021**, 11 (9), 4187–4206.
- Zipser, C. M.; Cragg, J. J.; Guest, J. D.; Fehlings, M. G.; Jutzeler, C. R.; Anderson, A. J.; et al. Cell-Based and Stem-Cell-Based Treatments for Spinal Cord Injury: Evidence from Clinical Trials. *Lancet Neurol.* **2022**, 21 (7), 659–670.
- Li, L.; Mu, J.; Zhang, Y.; Zhang, C.; Ma, T.; Chen, L.; et al. Stimulation by Exosomes from Hypoxia Preconditioned Human Umbilical Vein Endothelial Cells Facilitates Mesenchymal Stem Cells Angiogenic Function for Spinal Cord Repair. *ACS Nano* **2022**, 16 (7), 10811–10823.
- Fan, L.; Liu, C.; Chen, X.; Zheng, L.; Zou, Y.; Wen, H.; et al. Exosomes-Loaded Electroconductive Hydrogel Synergistically Promotes Tissue Repair after Spinal Cord Injury via Immunoregulation and Enhancement of Myelinated Axon Growth. *Adv. Sci.* **2022**, 9 (13), No. 2105586.
- Huang, L.; Fu, C.; Xiong, F.; He, C.; Wei, Q. Stem Cell Therapy for Spinal Cord Injury. *Cell Transplant.* **2021**, 30, No. 0963689721989266.
- Zha, K.; Yang, Y.; Tian, G.; Sun, Z.; Yang, Z.; Li, X.; et al. Nerve Growth Factor (NGF) and NGF Receptors in Mesenchymal Stem/Stromal Cells: Impact on Potential Therapies. *Stem Cells Transl. Med.* **2021**, 10 (7), 1008–1020.
- Shen, H.; Gu, X.; Wei, Z. Z.; Wu, A.; Liu, X.; Wei, L. Combinatorial Intranasal Delivery of Bone Marrow Mesenchymal Stem Cells and Insulin-like Growth Factor-1 Improves Neurovascularization and Functional Outcomes Following Focal Cerebral Ischemia in Mice. *Exp. Neurol.* **2021**, 337, No. 113542.
- Andrzejewska, A.; Dabrowska, S.; Lukomska, B.; Janowski, M. Mesenchymal Stem Cells for Neurological Disorders. *Adv. Sci.* **2021**, 8 (7), No. 2002944.
- Wang, C.-Z.; Chen, S.-M.; Chen, C.-H.; Wang, C.-K.; Wang, G.-J.; Chang, J.-K.; et al. The Effect of the Local Delivery of Alendronate on Human Adipose-Derived Stem Cell-Based Bone Regeneration. *Biomaterials* **2010**, 31 (33), 8674–8683.
- Religa, P. The Future Application of Induced Pluripotent Stem Cells in Vascular Regenerative Medicine. *Cardiovasc. Res.* **2012**, 96 (3), 348–349.
- Nombela-Arrieta, C.; Ritz, J.; Silberstein, L. E. The Elusive Nature and Function of Mesenchymal Stem Cells. *Nat. Rev. Mol. Cell Biol.* **2011**, 12 (2), 126–131.
- Qin, T.; Zhang, G.; Zheng, Y.; Li, S.; Yuan, Y.; Li, Q.; et al. A Population of Stem Cells with Strong Regenerative Potential Discovered in Deer Antlers. *Science* **2023**, 379 (6634), 840–847.
- Yao, B.; Wang, C.; Zhou, Z.; Zhang, M.; Zhao, D.; Bai, X.; et al. Comparative Transcriptome Analysis of the Main Beam and Brow Tine of Sika Deer Antler Provides Insights into the Molecular Control of Rapid Antler Growth. *Cell. Mol. Biol. Lett.* **2020**, 25 (1), 42.
- Bajpai, V. K.; Mistriotis, P.; Loh, Y.-H.; Daley, G. Q.; Andreadis, S. T. Functional Vascular Smooth Muscle Cells Derived from Human Induced Pluripotent Stem Cells via Mesenchymal Stem Cell Intermediates. *Cardiovasc. Res.* **2012**, 96 (3), 391–400.
- Wang, D.; Berg, D.; Ba, H.; Sun, H.; Wang, Z.; Li, C. Deer Antler Stem Cells Are a Novel Type of Cells That Sustain Full Regeneration of a Mammalian Organ—Deer Antler. *Cell Death Dis.* **2019**, 10 (6), 443.
- Rong, X.; Chu, W.; Zhang, H.; Wang, Y.; Qi, X.; Zhang, G.; et al. Antler Stem Cell-Conditioned Medium Stimulates Regenerative Wound Healing in Rats. *Stem Cell Res. Ther.* **2019**, 10 (1), 326.
- Lei, J.; Jiang, X.; Li, W.; Ren, J.; Wang, D.; Ji, Z.; Wu, Z.; Cheng, F.; Cai, Y.; Yu, Z.-R.; Belmonte, J. C. L.; Li, C.; Liu, G.-H.; Zhang, W.; Qu, J.; Wang, S. Exosomes from Antler Stem Cells Alleviate Mesenchymal Stem Cell Senescence and Osteoarthritis. *Protein Cell* **2022**, 13 (3), 220–226.
- Jin, S.; Wang, Y.; Wu, X.; Li, Z.; Zhu, L.; Niu, Y.; et al. Young Exosome Bio-Nanoparticles Restore Aging-Impaired Tendon Stem/Progenitor Cell Function and Replicative Capacity. *Adv. Mater.* **2023**, 35 (18), No. 2211602.
- Tan, F.; Li, X.; Wang, Z.; Li, J.; Shahzad, K.; Zheng, J. Clinical Applications of Stem Cell-Derived Exosomes. *Signal Transduction Targeted Ther.* **2024**, 9 (1), 17.
- Xia, B.; Gao, X.; Qian, J.; Li, S.; Yu, B.; Hao, Y.; et al. A Novel Superparamagnetic Multifunctional Nerve Scaffold: A Remote Actuation Strategy to Boost In Situ Extracellular Vesicles Production

- for Enhanced Peripheral Nerve Repair. *Adv. Mater.* **2024**, *36* (3), No. 2305374.
- (31) Han, M.; Yang, H.; Lu, X.; Li, Y.; Liu, Z.; Li, F.; et al. Three-Dimensional-Cultured MSC-Derived Exosome-Hydrogel Hybrid Microneedle Array Patch for Spinal Cord Repair. *Nano Lett.* **2022**, *22* (15), 6391–6401.
- (32) Nieto-Diaz, M. Deer Antler Innervation and Regeneration. *Front. Biosci.* **2012**, *17* (1), 1389.
- (33) S, S.; Camardo, A.; Dahal, S.; Ramamurthi, A. Surface-Functionalized Stem Cell-Derived Extracellular Vesicles for Vascular Elastic Matrix Regenerative Repair. *Mol. Pharmaceutics* **2023**, *20* (6), 2801–2813.
- (34) Wu, W.; Jia, S.; Xu, H.; Gao, Z.; Wang, Z.; Lu, B.; et al. Supramolecular Hydrogel Microspheres of Platelet-Derived Growth Factor Mimetic Peptide Promote Recovery from Spinal Cord Injury. *ACS Nano* **2023**, *17* (4), 3818–3837.
- (35) Yano, S.; Tazawa, H.; Kagawa, S.; Fujiwara, T.; Hoffman, R. M. FUCCI Real-Time Cell-Cycle Imaging as a Guide for Designing Improved Cancer Therapy: A Review of Innovative Strategies to Target Quiescent Chemo-Resistant Cancer Cells. *Cancers* **2020**, *12* (9), 2655.
- (36) Koh, S.-B.; Mascaldi, P.; Rodriguez, E.; Lin, Y.; Jodrell, D. I.; Richards, F. M.; et al. A Quantitative FastFUCCI Assay Defines Cell Cycle Dynamics at a Single-Cell Level. *J. Cell Sci.* **2017**, *130* (2), 512–520.
- (37) Geng, H.; Li, Z.; Li, Z.; Zhang, Y.; Gao, Z.; Sun, L.; et al. Restoring Neuronal Iron Homeostasis Revitalizes Neurogenesis after Spinal Cord Injury. *Proc. Natl. Acad. Sci. U. S. A.* **2023**, *120* (46), No. e2220300120.
- (38) Tran, A. P.; Warren, P. M.; Silver, J. The Biology of Regeneration Failure and Success After Spinal Cord Injury. *Physiol. Rev.* **2018**, *98* (2), 881–917.
- (39) Li, S.; Wang, P.; Ye, M.; Yang, K.; Cheng, D.; Mao, Z.; et al. Cysteine-Activatable Near-Infrared Fluorescent Probe for Dual-Channel Tracking Lipid Droplets and Mitochondria in Epilepsy. *Anal. Chem.* **2023**, *95* (11), 5133–5141.
- (40) Mathew, B.; Ravindran, S.; Liu, X.; Torres, L.; Chennakesavalu, M.; Huang, C.-C.; et al. Mesenchymal Stem Cell-Derived Extracellular Vesicles and Retinal Ischemia-Reperfusion. *Biomaterials* **2019**, *197*, 146–160.
- (41) Ciani, L.; Salinas, P. C. WNTs in the Vertebrate Nervous System: From Patterning to Neuronal Connectivity. *Nat. Rev. Neurosci.* **2005**, *6* (5), 351–362.
- (42) Feltri, M. L.; Poitelon, Y. HIPPO Stampede in Nerve Sheath Tumors. *Cancer Cell* **2018**, *33* (2), 160–161.
- (43) Tian, X.; Hou, W.; Bai, S.; Fan, J.; Tong, H.; Bai, Y. XAV939 Promotes Apoptosis in a Neuroblastoma Cell Line via Telomere Shortening. *Oncol. Rep.* **2014**, *32* (5), 1999–2006.
- (44) Barrette, A. M.; Ronk, H.; Joshi, T.; Mussa, Z.; Mehrotra, M.; Bouras, A.; et al. Anti-Invasive Efficacy and Survival Benefit of the YAP-TEAD Inhibitor Verteporfin in Preclinical Glioblastoma Models. *Neuro-Oncol.* **2022**, *24* (5), 694–707.
- (45) Liu, H.; Li, R.; Liu, T.; Yang, L.; Yin, G.; Xie, Q. Immunomodulatory Effects of Mesenchymal Stem Cells and Mesenchymal Stem Cell-Derived Extracellular Vesicles in Rheumatoid Arthritis. *Front. Immunol.* **2020**, *11*, 1912.
- (46) Xiong, T.; Yang, K.; Zhao, T.; Zhao, H.; Gao, X.; You, Z.; et al. Multifunctional Integrated Nanozymes Facilitate Spinal Cord Regeneration by Remodeling the Extrinsic Neural Environment. *Adv. Sci.* **2023**, *10* (7), No. 2205997.
- (47) Wang, L.; Wang, D.; Ye, Z.; Xu, J. Engineering Extracellular Vesicles as Delivery Systems in Therapeutic Applications. *Adv. Sci.* **2023**, *10* (17), No. 2300552.
- (48) Miranpuri, G. S.; Schomberg, D. T.; Alrfai, B.; King, K. C.; Rynearson, B.; Wesley, V. S.; et al. Role of Matrix Metalloproteinases 2 in Spinal Cord Injury-Induced Neuropathic Pain. *Ann. Neurosci.* **2016**, *23* (1), 25–32.
- (49) Shen, K.; Sun, G.; Chan, L.; He, L.; Li, X.; Yang, S.; et al. Anti-Inflammatory Nanotherapeutics by Targeting Matrix Metalloproteases for Immunotherapy of Spinal Cord Injury. *Small* **2021**, *17* (41), No. 2102102.
- (50) Zhu, B.; Gu, G.; Ren, J.; Song, X.; Li, J.; Wang, C.; et al. Schwann Cell-Derived Exosomes and Methylprednisolone Composite Patch for Spinal Cord Injury Repair. *ACS Nano* **2023**, *17* (22), 22928–22943.
- (51) Yang, J.; Yang, K.; Man, W.; Zheng, J.; Cao, Z.; Yang, C.-Y.; et al. 3D Bio-Printed Living Nerve-like Fibers Refine the Ecological Niche for Long-Distance Spinal Cord Injury Regeneration. *Bioact. Mater.* **2023**, *25*, 160–175.
- (52) Goodman, Z. D. Grading and Staging Systems for Inflammation and Fibrosis in Chronic Liver Diseases. *J. Hepatol.* **2007**, *47* (4), 598–607.
- (53) Qin, T.; Li, C.; Xu, Y.; Qin, Y.; Jin, Y.; He, R.; et al. Local Delivery of EGFR+NSCs-Derived Exosomes Promotes Neural Regeneration Post Spinal Cord Injury via miR-34a-5p/HDAC6 Pathway. *Bioact. Mater.* **2024**, *33*, 424–443.
- (54) Nie, H.; Jiang, Z. Bone Mesenchymal Stem Cell-Derived Extracellular Vesicles Deliver microRNA-23b to Alleviate Spinal Cord Injury by Targeting Toll-like Receptor TLR4 and Inhibiting NF- κ B Pathway Activation. *Bioengineered* **2021**, *12* (1), 8157–8172.
- (55) Welsh, J. A.; Goberdhan, D. C. I.; O'Driscoll, L.; Buzas, E. I.; Blenkiron, C.; Bussolati, B.; et al. Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches. *J. Extracell. Vesicles* **2024**, *13* (2), No. e12404.
- (56) Piccolo, S.; Dupont, S.; Cordenonsi, M. The Biology of YAP/TAZ: Hippo Signaling and Beyond. *Physiol. Rev.* **2014**, *94* (4), 1287–1312.
- (57) Cassani, M.; Fernandes, S.; Oliver-De La Cruz, J.; Durikova, H.; Vrbsky, J.; Patočka, M.; et al. YAP Signaling Regulates the Cellular Uptake and Therapeutic Effect of Nanoparticles. *Adv. Sci.* **2024**, *11* (2), No. 2302965.
- (58) Pham, T. C.; Jayasinghe, M. K.; Pham, T. T.; Yang, Y.; Wei, L.; Usman, W. M.; et al. Covalent Conjugation of Extracellular Vesicles with Peptides and Nanobodies for Targeted Therapeutic Delivery. *J. Extracell. Vesicles* **2021**, *10* (4), No. e12057.
- (59) Théry, C.; Witwer, K. W.; Aikawa, E.; Alcaraz, M. J.; Anderson, J. D.; Andriantsitohaina, R.; et al. Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* **2018**, *7* (1), No. 1535750.
- (60) Courtine, G.; Sofroniew, M. V. Spinal Cord Repair: Advances in Biology and Technology. *Nat. Med.* **2019**, *25* (6), 898–908.
- (61) Skinnider, M. A.; Gautier, M.; Teo, A. Y. Y.; Kathe, C.; Hutson, T. H.; Laskaratos, A.; et al. Single-Cell and Spatial Atlases of Spinal Cord Injury in the Tabulae Paralytica. *Nature* **2024**, *631* (8019), 150–163.
- (62) Lener, T.; Gimona, M.; Aigner, L.; Börger, V.; Buzas, E.; Camussi, G.; et al. Applying Extracellular Vesicles Based Therapeutics in Clinical Trials – an ISEV Position Paper. *J. Extracell. Vesicles* **2015**, *4* (1), 30087.
- (63) Elvira, K. S. Microfluidic Technologies for Drug Discovery and Development: Friend or Foe? *Trends Pharmacol. Sci.* **2021**, *42* (7), 518–526.
- (64) Gao, X.; Li, S.; Yang, Y.; Yang, S.; Yu, B.; Zhu, Z.; et al. A Novel Magnetic Responsive miR-26a@SPIONs-OECs for Spinal Cord Injury: Triggering Neural Regeneration Program and Orienting Axon Guidance in Inhibitory Astrocytic Environment. *Adv. Sci.* **2023**, *10* (32), No. 2304487.
- (65) Khan, N. Z.; Cao, T.; He, J.; Ritzel, R. M.; Li, Y.; Henry, R. J.; et al. Spinal Cord Injury Alters microRNA and CD81+ Exosome Levels in Plasma Extracellular Nanoparticles with Neuroinflammatory Potential. *Brain. Behav. Immun.* **2021**, *92*, 165–183.
- (66) Tran, N. H.; Qiao, R.; Xin, L.; Chen, X.; Liu, C.; Zhang, X.; et al. Deep Learning Enables de Novo Peptide Sequencing from Data-Independent-Acquisition Mass Spectrometry. *Nat. Methods* **2019**, *16* (1), 63–66.